

A Taxonomic and Phylogenetic Investigation of Conifer Endophytes
of Eastern Canada

by

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Abstract

Research interest in endophytic fungi has increased substantially, yet is the current research paradigm capable of addressing fundamental taxonomic questions? More than half of the ca. 30,000 endophyte sequences accessioned into GenBank are unidentified to the family rank and this disparity grows every year. The problems with identifying endophytes are a lack of taxonomically informative morphological characters *in vitro* and a paucity of relevant DNA reference sequences. A study involving ca. 2,600 *Picea* endophyte cultures from the Acadian Forest Region in Eastern Canada sought to address these taxonomic issues with a combined approach involving molecular methods, classical taxonomy, and field work. It was hypothesized that foliar endophytes have complex life histories involving saprotrophic reproductive stages associated with the host foliage, alternative host substrates, or alternate hosts. Based on inferences from phylogenetic data, new field collections or herbarium specimens were sought to connect unidentifiable endophytes with identifiable material. Approximately 40 endophytes were connected with identifiable material, which resulted in the description of four novel genera and 21 novel species and substantial progress in endophyte taxonomy. Endophytes were connected with saprotrophs and exhibited reproductive stages on non-foliar tissues or different hosts. These results provide support for the foraging ascomycete hypothesis, postulating that for some fungi endophytism is a secondary life history strategy that facilitates persistence and dispersal in the absence of a primary host. This thesis provides an alternate approach to identifying endophytes by considering the whole fungus interacting within its natural habitat.

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My mind was, as it still is, a vagabond easily distracted from one thing to another. And then thought never stops either. The end of the last page is only the beginning of another chapter, and every peak attained is but the spring-board for a new leap.

• • •

But as yet I have hardly begun. A child is born when he is nine months old, a man when he is thirty. The blossom has come and gone, but the fruit has to ripen before it rots on the tree.

Giovanni Papini, *Un uomo finito*

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General introduction

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Project background

The Acadian Forest is the dominant forest region in the Canadian Maritime provinces, including elements of the neighbouring Eastern Deciduous Forest and Boreal Forest Regions. Late-successional forest types are composed of the defining species *Picea rubens*, and other long-lived, shade tolerant species including *Acer saccharum*, *Betula alleghaniensis*, *Fagus grandifolia*, *Pinus strobus*, and *Tsuga canadensis* (Mosseler et al. 2003a). Natural climax forest dynamics are generally driven by small gap-producing events, such as insect-, disease-, or wind-caused mortality, with stand-replacing disturbances being rare (Lorimer 1977, Wein and Moore 1977).

However, European settlement brought intensive deforestation activity and large-scale fire disturbances, which resulted in the disruption of normal forest dynamics. Additional disruptive human activities include persistent selective harvesting for *Picea*, conversion of mixed-wood forests to *Abies balsamea*-dominated conifer forests, pesticide spraying, and fire suppression (Blais 1983, Forbes et al. 1998, Loo and Ives 2003). These changes to the forest ecosystem result in more extensive even-aged *Abies-Picea* forests and a decline in *Picea rubens* (Gordon 1996), while increasing the susceptibility of forests to various pests and pathogens.

The eastern spruce budworm (*Choristoneura fumiferana*) is one of the most destructive forest pests in Eastern Canada, capable of causing substantial decline in vigour and increased mortality in *Abies balsamea* and *Picea* species. The last epidemic in eastern North America (1970s) resulted in substantial ecological disturbance and economic loss. Budworm outbreaks cause a significant loss of timber volume, an

increase in forest fires, substantial CO₂ emissions, and are currently increasing in frequency, extent, and severity as a result of climate variability and anthropogenic changes to the forest ecosystem (Blais 1983, Fleming et al. 2002, Dymond et al. 2010). Budworm outbreaks have occurred at periodic intervals of about 35 years for the last three centuries, with populations and subsequent defoliation levels currently on the rise (Royama 1984). In Quebec, almost 3.4 million hectares were moderately to severely defoliated by the budworm in 2014 alone, roughly equal to the total number of hectares defoliated in the province from 1987–2007 (National Forestry Database 2016b; <http://nfdp.ccfm.org/>). Chang et al. (2012) estimate that from 2012–2014, uncontrolled moderate budworm outbreaks could cost the New Brunswick economy CDN\$3.3 billion and severe budworm outbreaks \$4.7 billion.

The driving factors initiating budworm outbreak cycles have not been elucidated, although epidemiological studies contradict the hypothesis of one epicenter spreading across the region and suggest that populations across the landscape cycle in unison (Royama 1984, Royama et al. 2005). Royama (1984) considered this basic cycle to be determined by various density-dependent mortality factors during the third to sixth larval instars: parasitism, predation, food shortage, weather, and the “fifth agent”. This last undefined variable was hypothesized to be a complex of cryptic causes, which may explain observed mortality resulting from unclear symptoms. Royama deduced that population oscillation was ultimately driven by parasitism and the fifth agent.

Motivated by Royama’s intriguing “fifth agent” and the importance of the survival of feeding larvae in driving regional outbreaks, research was initiated under the

hypothesis that toxigenic fungal endophytes represented this unknown variable (Miller et al. 1985). This has become a long-term collaborative effort resulting in the discovery of antiinsectan metabolites produced by fungi inhabiting healthy conifer needles that can remediate the negative effects of eastern spruce budworm (Sumarah et al. 2010, Sumarah et al. 2015). These fungi, called endophytes, can protect the host plant by reducing the severity of grazing by insect pests (Vega 2008). This research program, in affiliation with J.D. Irving Ltd., is resulting in the large-scale planting of conifer seedlings inoculated with native endophyte strains that produce antiinsectan toxins. The goal of this new approach is to decrease the probability of a major budworm epidemic and limit the damage when one occurs. Several endophyte strains are undergoing field tests in New Brunswick to test their efficacy in limiting budworm damage.

One of the main challenges identified early in this research program is the difficulty in identifying endophytes. Endophytes are frequently sterile in culture and therefore cannot be reliably identified on the basis of taxonomically informative morphological characters involved in sporulation. Conifer endophytes are also phylogenetically diverse, belonging to large taxonomic orders (e.g.: Helotiales, Rhytismatales) that are severely underrepresented in public databases and the scientific literature by reference sequences, for example the internal transcribed spacer (ITS) barcode. The consequence is an inability to confidently identify many endophytes using either cultural characters or sequence data. The absence of such taxonomic reference sequence data has resulted in >30,000 fungal endophyte ITS sequences in GenBank, the vast majority of which are unidentified even to family.

What is an endophyte?

Endophytes are an artificial assemblage of phylogenetically diverse fungi that can asymptotically infect and inhabit healthy plant tissue. Endophyte research interest was propelled by the discovery of the clavicipitaceous (Clavicipitaceae, Hypocreales) endophytes of cool-season grasses, which produce toxic alkaloids associated with livestock toxicoses and increased tolerance to insect pests (Clay 1988, Siegel et al. 1990). These endophytes systemically infect their hosts and may increase growth and seed production and protect or confer resistance or tolerance to the host from a variety of abiotic and biotic stresses such as drought, heat, waterlogging, heavy metal toxicity, herbivory, and fungal pathogens (Rice et al. 1990, Monnet et al. 2001, Kuldau and Bacon 2008, Song et al. 2015, Xia et al. 2015). Because of their economic importance for livestock agriculture and their potential use as inoculants of turfgrass against insects, clavicipitaceous endophytes received much attention and consequently shaped the endophyte research paradigm.

However, the clavicipitaceous endophyte model is not necessarily applicable to foliar endophytes of woody plants. Instead of a phylogenetically-coherent group of taxa which display host-specific, systemic, vertically-transmitted life strategies, foliar endophytes of woody plants are phylogenetically very diverse, frequently exhibit very low host preference, are euryoecious, and are horizontally-transmitted. This phylogenetic diversity and lack of understanding of life histories have resulted in a significant knowledge gap and leads to the question, “What is an endophyte?”

While the definition of the term endophyte used to introduce this section is ambiguous, it is perhaps the most suitable because of its inclusiveness and lack of assumptions. The concept of what an endophyte is and the subsequent functional or conceptual categorization of these organisms may be more readily perceived for clavicipitaceous endophytes; however the concept becomes unclear when the non-clavicipitaceous endophytes of woody plants are taken into account. Narrow definitions may omit significant numbers of species that clearly exhibit key characteristics of endophytism or pigeonhole endophytes into artificial functional groups based on limited knowledge (Rodriguez et al. 2009).

The definition of what constitutes an endophyte has changed over time (e.g.: Wilson 1995, Stone et al. 2000, Schulz and Boyle 2006, Hyde and Soyong 2008) and is often a function of what characters are emphasized by the investigator, which may include host specificity or evidence of mutualism. Rogers (2000) adopted a narrow concept of endophytism and did not consider fungi with cryptic or latent phases on hosts followed by eventual sporulation to be endophytes, rather he considered them latent pathogens. Endophytes undoubtedly operate along a continuum from mutualism to antagonism, which is driven by a multitude of fungus-host interactions. Difficulties with compartmentalizing species by apparent latent pathogenicity or mutualism may be exemplified by the genus *Phialocephala*, one of the major groups studied in this thesis. *Phialocephala* contains many of the so-called dark septate endophytes, species that are ubiquitous in above-ground and below-ground tissues of an extreme variety of hosts over a wide geographic range in the northern hemisphere. Fungal-host interactions

range from pathogenic (Wilcox and Wang 1987, Stoyke and Currah 1993, Tellenbach et al. 2011) to neutral or mutualistic (Vohník et al. 2005, Peterson et al. 2008, Newsham 2011, Otgonsuren and Lee 2012, Tellenbach and Sieber 2012), and appear to be strain-dependent and not correlated with species (Currah et al. 1993, Tellenbach et al. 2011). Does *Phialocephala fortinii* conform to an exclusive definition of endophytism or can only certain strains, in certain conditions, considered to be so? For example, Alvarez-Loayza et al. (2011) discovered the light-induced pathogenicity of *Diplodia mutila*, a common asymptomatic endophyte of mature *Iriartea deltoidea* trees and reported pathogen of *I. deltoidea* seedlings. While Mostert (2000) felt that endophytes should never provoke visible disease symptoms, it is evident that the demarcation of endophytism cannot be constrained by the presence of mutualism when such an attribute is conditional.

Wilson (1995) redefined endophytism by placing importance on the asymptomatic infection of plant tissue, but he considered endophytism to be more of a strategic phase in the life history of a fungus rather than an obligate strategy. Thus, the latent or dormant asymptomatic infection of a host by a pathogen would represent an endophytic phase, regardless of the imminent disease symptoms. This holistic approach better acknowledges the complex relationships between the non-clavicipitaceous endophytes and their hosts and does not carry preconceptions of unconditional or persistent mutualism. Thus the definition provided by Wilson (1995) is adopted for this thesis: “Endophytes are fungi which, for all or part of their life cycle, invade the tissues

of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease”.

Conifer endophytes: ubiquitous, complex, and diverse

In natural stands, virtually all conifer needles are infected by endophytes, with infections increasing with needle age (Bernstein and Carroll 1977, Stone 1987, Espinosa-Garcia and Langenheim 1990). For example, Barklund (1987) reported infection rates of 86–95% by *Lophodermium piceae* alone in three-year-old *Picea abies* needles from healthy stands in Sweden. McCutcheon et al. (1993) investigated *Rhabdocline parkeri* genotypes in *Pseudotsuga menziesii* needles and estimated the total number of needle infections in a single old growth tree to be 1×10^{11} , represented by up to 1000 different genotypes. Foliar endophytes of conifers are ubiquitous and phylogenetically diverse, and their functional roles in the forest ecosystem are largely unknown. The production of antimicrobial and antiinsectan secondary metabolites by conifer endophyte strains from the Acadian Forest Region suggests hosts may be conferred some tolerance to attack by pathogens and pests (Miller et al. 2008, Sumarah et al. 2010, McMullin et al. 2015, Richardson et al. 2015, Tanney et al. 2016b).

Conifer endophytes show varying host and substrate preferences or specificity. For example, *Lophodermium piceae* is a common European *Picea* endophyte that only produces its ascomata on senescent or dead needles (Müller and Hallaksela 1998, Stefani and Bérubé 2006, Sokolski et al. 2007) while the sexual structures of commonly reported *Picea* endophytes such as *Hypoxylon* spp. and *Nemania serpens* are primarily thought of as saprotrophs of decaying hardwood (Stefani and Bérubé 2006, Sokolski et

al. 2007). *Phialocephala scopiformis*, described as a *Picea abies* periderm endophyte in Germany, is a common foliar endophyte of *Picea* in the Acadian Forest Region of Eastern Canada that produces apothecia on decaying *Picea* branches (Kowalski and Kehr 1995, Tanney et al. 2016a). *Dwayaangam colodena*, an aquatic hyphomycete in boreal and mixed-wood forest streams, is also a common needle endophyte of *Picea mariana* and *Picea rubens* and is reported from rainwater collected from *Picea abies*, *Pinus sylvestris*, and *Vaccinium myrtillus* in Europe (Sokolski et al. 2006a, Sokolski et al. 2006b, Révay and Gönczöl 2010, Sumarah et al. 2010). The discovery of an aquatic hyphomycete conifer endophyte and reports of hardwood saprotrophs as conifer endophytes are evocative of more complex interactions between endophytes and their environment.

Xylariaecous (Xylariaceae, Xylariales) endophytes are detected in varying abundance in most endophyte studies involving woody plants, regardless of geographic location or host (Dreyfuss and Petrini 1984, Petrini and Petrini 1985, Davis et al. 2003, Okane et al. 2008, U'Ren et al. 2016). This ubiquity and apparent host-neutrality has led to much interest and speculation into the role of endophytism in fungal ecology. In this context, Carroll (1999) posited the foraging ascomycete hypothesis, suggesting that for some saprotrophs endophytism is a secondary life-history strategy facilitating persistence and dispersal in the absence of a primary host. This latent saprotroph strategy may involve a fungus persisting as a quiescent thallus for some time until the host tissue senesces or dies, at which point the expansive colonization of the fungus into the host substrate ensues. The fungus may cycle through foliage as an endophyte for some time until it comes into contact with a suitable primary host and sexual

reproduction or another facet of its life history is permitted. A fungus capable of endophytically infecting host foliage is afforded a refuge buffering it from UV radiation, drought, starvation, competition, mycophagy, and other stresses, while being provided with a source of nutrition and a vehicle for persistence and dispersal (Thomas et al. 2016). The foraging ascomycete hypothesis might explain why endophytism is common throughout Dikarya and it may also provide a strategy to identify unknown endophytes, one that involves considering the broader ecology of endophytes.

Problems identifying endophytes

Culture-dependent endophyte studies involve rigorous sterilization of plant tissue surfaces to kill non-endophytic fungi (e.g.: epiphytes or viable propagules) and other organisms. The surface sterilized plant tissue is then cut into segments, placed on agar media (e.g.: 2% malt extract agar; MEA), and incubated. Mycelia emerging from plant tissue segments are excised and subcultured to generate axenic cultures. Capturing more biodiversity from plant tissue can involve the addition of other methods such as enzymatic digestion of plant tissue, use of non-standard media, or higher throughput protocols (Prior et al. 2014, Greenfield et al. 2015).

Traditional endophyte studies relied upon morphological characters to distinguish, group, and identify endophyte strains. Identification to genus or species is feasible when fungi produce taxonomically informative morphological characters such as conidiomata or ascomata in vitro. Inducing sporulation in sterile cultures is a laborious process with varying success that involves prolonged incubation under various culture conditions, with treatments including different artificial and natural media, light

regimes, incubation temperatures, gradual desiccation, mechanical stimulation, flotation in still or aerated water, and the addition of natural substrates (e.g.: sterilized plant leaves) (Sokolski et al. 2006a, Knapp et al. 2015, Tanney et al. 2016a). Recalcitrant sterile cultures are generally unidentifiable because hyphae typically lack morphological characters allowing for precise taxonomic designation; in such examples, unidentified endophytes can be grouped by codes or description according to morphotypes (e.g.: “sterile white” and “sterile pigmented” in Johnson and Whitney 1989). The absence of precisely identified strains, especially for strains not accessioned in culture collections, can result in the inability to make meaningful conclusions or comparisons between traditional and modern studies.

Previous attempts to identify specific sterile endophytes included the use of a specific absorbed immunoserum with on-section immunogold labeling to identify *Lophodermium piceae* hyphae in symptomless *Picea abies* needles by immunoelectron microscopy (Suske and Acker 1989). However, the most important development in the categorization and identification of sterile endophyte strains was the use of molecular DNA tools for fingerprinting and barcoding (Leuchtman and Clay 1990, Haemmerli et al. 1992). The advent of next generation sequencing (NGS) technology now enables the amplification of endophyte fungal DNA directly from plant tissue, potentially bypassing the labor and selective biases associated with culture-dependent methods and capturing millions of reads at a relatively low cost.

Despite these advances, an analogous problem that plagued pre-molecular culture-based studies still confronts both culture-dependent and culture-independent

studies relying on DNA sequence-based identification: the precise identification of endophyte strains or sequences lacking taxonomically informative data. Most contemporary endophyte studies use the ITS barcode to facilitate identification or grouping of endophyte strains. When a fungus belonging to a species, genus, or even family that is unrepresented by an accurately identified reference sequence in an accessible DNA sequence repository, such as NCBI GenBank or UNITE, its identification using DNA sequences may be irresolvable. The inability to identify endophyte sequences yields results similar to that of traditional studies, however instead of “sterile white” and “sterile pigmented” there are “fungal endophyte sp.” and “Ascomycota sp.”. Accordingly, many endophyte studies report endophyte taxa that are often unidentified to species or even genus (Arnold and Lutzoni 2007, U’Ren et al. 2012, Huang et al. 2016).

However, unidentified endophyte DNA sequences accessioned in GenBank and other sequence repositories are a valuable resource. Comparison of unidentified sequences connected with biogeographic data can provide insight into endophyte ecology despite the absence of a binomial name. These sequences can also be clustered into OTUs and presented as putative species hypotheses (Abarenkov et al. 2010). The ability to cluster OTUs into species hypotheses with associated aggregate data has led some mycologists to advocate a sequence-based taxonomic system allowing for the description of fungi based solely on NGS data (Hibbett and Taylor 2013, Taylor and Hibbett 2013). This movement is partly justified by the growing number of unidentified sequences that probably represent a portion of the currently unknown fungal

biodiversity. Names provide consistent identifiers that facilitate the communication of taxonomic concepts; for example, the name *Aspergillus fumigatus* provides a wealth of associated information compared with a unique digital identifier from GenBank.

Endophyte species identification is not constrained by the ability to generate appropriate data but by the dearth of taxonomists and the current approach to this taxonomic problem. If sterile endophyte strains or endophyte NGS cannot be identified by ITS barcodes and other sequences because of a lack of reference data, then the clear solution is to expand upon the available reference sequences.

How to identify endophytes

Figure 1 illustrates different scenarios and approaches to identifying an unknown *Picea* endophyte, using *Micraspis acicola* as an example:

- 1) Healthy needles are surface sterilized, aseptically cut into segments, placed on the surface of an agar medium (e.g.: MEA), and incubated. An axenic culture is generated by excising and subculturing mycelium emerging from a needle section. Sporulation occurs or is induced, providing taxonomically informative characters that result in its identification as *Micraspis acicola*. Named reference sequences can be created from this identified culture.
- 2) The axenic culture is sterile. Sequencing the ITS barcode and a subsequent NCBI GenBank BLAST search provide a match with an accessioned sequence named *Micraspis acicola*.
- 3) Fungal DNA is extracted from the needles and ITS barcodes are amplified using NGS (e.g.: Illumina MiSeq platform). A bioinformatics pipeline processes raw

sequence reads and designates OTUs. *Micraspis acicola* OTUs are identified using methods such as pairwise comparisons or lowest common ancestor analyses mapping sequences onto nodes on a reference taxonomic tree (Buee et al. 2009).

- 4) Sequence does not result in an identification; e.g.: 100% match with unidentified endophyte sequence ("*Picea mariana* Fungal Endophyte sp.") or no similar sequences present in database.
- 5) Field specimens of *Micraspis acicola* conidiomata or ascomata are collected in the field during a broad survey of *Picea*-associated fungi. Axenic cultures generated from conidia or ascospores are used to generate reference sequences, which can be used to identify endophytes (e.g.: unidentified OTUs in scenario #4). Alternatively, reference sequences can be generated from herbarium specimens or cultures accessioned in collections.

The strategy employed in this thesis is straightforward: culturable endophytes that are unidentifiable based on current sequence data or an absence of taxonomically informative characters are identified by connecting them to identifiable specimens using DNA barcodes. Specimens of named-but-unsequenced or undescribed species containing taxonomically informative characters are inferred to represent saprotrophic reproductive states of unidentified endophytes based on hypotheses derived from phylogenetic or taxonomic evidence. The resulting DNA barcodes generated from identifiable herbarium specimens, new field collections, and sporulating cultures are used to connect unidentified endophytes with names.

Overview of thesis

In this thesis, the approach described in Fig. 1 is used to investigate the taxonomy and phylogeny of culturable foliar endophytes from *Picea rubens* and other conifer trees from the Acadian Forest Region of Eastern Canada. I provide an examination of the general endophyte biodiversity of *Picea* and select certain groups for more detailed investigation. In the first chapter, I demonstrate the ability to connect endophytes with their respective saprotrophic life stages in *Phialocephala* (Mollisiaceae, Helotiales). These results also include the first verified discovery of sexual states in *Phialocephala*, the epitypification of *P. dimorphospora* and subsequent delineation of *Phialocephala* s.s., and the description of four novel species, one new combination, and previously undocumented synanamorphs.

The phylogeny of Mollisiaceae, a poorly understood family of discomycetes usually considered as a saprobe of woody debris, is explored in chapter two using five genetic loci and material derived from new field collections, culture collections, and herbaria. Three potential phylogenetic markers and secondary barcodes are tested, taxonomic and nomenclatural issues involving Mollisiaceae are explored, and the descriptions of ten novel species are presented. The unexpected prevalence of endophytism throughout Mollisiaceae is investigated.

In chapter three, I describe *Diaporthe maritima*, one of the most commonly isolated *Picea* endophytes from New Brunswick, supported by morphological observations and the application of the genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al. 2000) concept using four unlinked loci. Four antifungal

and antiinsectan metabolites are characterized from *D. maritima* strains through a collaborative effort with natural product chemists.

Picea rubens endophytes in Phacidiaceae (Phacidiales), a group of conifer needle fungi with a rich classical taxonomic history but little presence in DNA sequence databases, are characterized morphologically and phylogenetically in chapter four. This results in the identification of seven species including two novel *Phacidium* species and three novel monotypic genera described to accommodate distinct species. This chapter considerably increases the known biodiversity of conifer-associated Phacidiaceae species; previous reports of Phacidiaceae species on Canadian conifers were scarce and species were typically considered pathogens (Farr and Rossman 2016).

In chapter five, the overall biodiversity of *Picea* endophytes isolated in New Brunswick is explored and evidence connecting unidentifiable endophytes with identifiable saprotrophic reproductive stages from field or herbarium specimens is presented. I describe three novel species including one novel genus, and discuss the general biology and ecology of conifer endophytes. The major endophyte families involved in this study include Mollisiaceae, Phacidiaceae, Rhytismataceae, and Xylariaceae, which are each comprised of hundreds of species and poorly represented by sequence data. Identified endophytes include species and genera that are known to classical taxonomy but not included in sequence databases (i.e.: named-but-unsequenced).

In the concluding section, I relate the above findings to the foraging ascomycete hypothesis and suggest future research directions for endophyte taxonomy.

Chapter 1: Sexual and asexual states of some endophytic

Phialocephala species of *Picea*

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ABSTRACT

Unidentified DNA sequences in culture-dependent or culture-independent studies of conifer endophytes are a persistent problem that requires a field approach to resolve. An investigation of foliar endophytes of *Picea glauca*, *P. mariana*, *P. rubens* and *Pinus strobus* in Eastern Canada, using a combined field, morphological, cultural and DNA sequencing approach, resulted in the frequent isolation of *Phialocephala* spp. and the first verified discovery of their mollisia-like sexual states in the field. *Phialocephala scopiformis* and *Ph. piceae* were the most frequent species isolated as endophytes from healthy conifer needles. Corresponding *Mollisia* or mollisioid sexual states for *Ph. scopiformis*, *Ph. piceae*, and several undescribed species in a clade containing *Ph. dimorphospora* were collected in the sampling area and characterized by analysis of the nuc internal transcribed spacer rDNA internal transcribed spacer (ITS) and gene for the largest subunit of RNA polymerase II (*RPB1*) loci. Four novel species and one new combination in a clade containing *Ph. dimorphospora*, the type of *Phialocephala*, are presented, accompanied by descriptions of apothecia and previously undocumented synanamorphs. An epitype culture and corresponding reference sequences for *Phialocephala dimorphospora* are proposed. The resulting ITS barcodes linked with robust taxonomic species concepts are an important resource for future research on forest ecosystems and endophytes.

KEYWORDS

DSE; dematiaceous hyphomycetes; endophytes; *Helotiales*; *Mollisia*; Vibrissaceae

INTRODUCTION

Kendrick (1961b) introduced the dematiaceous hyphomycete genus *Phialocephala* for a group of morphologically distinct species previously classified in *Leptographium*. He distinguished the genera based on their modes of enteroblastic, basipetal conidiogenesis; *Phialocephala* species have a stationary conidiogenous locus that gradually thickens as successive conidia are produced (phialides), while *Leptographium* species have a progressive locus that extends percurrently after each conidium, leaving an annellated zone (annelides). The type species, *Ph. dimorphospora*, was collected from rotten hardwood (*Ulmus* sp.) in Ontario, Canada and characterized by dark-pigmented penicillate conidiophores and phialides with deep collarettes yielding both ovoid primary conidia and globose secondary conidia that aggregate into slimy droplets. Subsequent to the inception of the genus, 37 *Phialocephala* species were described. Several were transferred out of *Phialocephala* (e.g. *Sporendocladia bactrospora*, *S. follicola*, *S. ivoriensis*, *S. truncata*) based on morphological and phylogenetic data (Jacobs et al. 2001, Day et al. 2012). *Phaeomollisia piceae* was recently combined into *Phialocephala* based on phylogenetic placement within a major clade of *Phialocephala* (Johnston et al. 2014), resulting in 29 currently accepted species in this genus (Wong et al. 2015, Index Fungorum 2016a).

Phialocephala-like asexual states occur across a moderately large lineage including the core clade of *Phialocephala* sensu stricto (s.s., *Ph. dimorphospora* and allied taxa; e.g.: Menkis et al. 2004, Grünig et al. 2009); the *Ph. fortinii* sensu lato (s.l.)–

Acephala applanata complex (PAC) and allied taxa; *Vibrissea* s.s. (Descals and Sutton 1976, Hamad and Webster 1988); and several more distantly related *Phialocephala* species (e.g. *Ph. scopiformis*, *Ph. sphaeroides*). *Phialocephala*-like anamorphs are also known from several unsequenced mollisoid taxa (e.g.: Le Gal and Mangenot 1956, Aebi 1972) or aquatic synanamorphs (e.g.: Webster and Descals 1975, Descals and Sutton 1976, Descals and Webster 1982). A sterile morphotype generic concept, *Acephala*, was established for *A. applanata*, a species frequently co-isolated with *Ph. fortinii* s.l. (Grünig and Sieber 2005). *Acephala* currently comprises two species and the generic name has been applied informally to a phylogenetically diverse range of sterile strains apparently congeneric with *Phialocephala* (Grünig et al. 2009, Münzenberger et al. 2009). The current taxonomic concept of *Phialocephala* is therefore polyphyletic, but with most sequenced species within a lineage encompassing the *Vibrissea–Loramycetes–Mollisia* clades sensu Wang et al. (2006a, 2006b) and Grünig et al. (2009).

Phialocephala species and allied taxa are often isolated from roots and decayed wood in heathlands, forests, and alpine ecosystems across north temperate and boreal regions, and as a group they display remarkable ecological plasticity (Addy et al. 2000, Grünig et al. 2009). The most studied belong to the PAC, which are frequently isolated from the cortex of primary roots and the periderm of lignified roots of ericaceous plants (e.g. *Calluna vulgaris*, *Empetrum nigrum*, *Vaccinium myrtillus*) and coniferous trees (e.g. *Abies*, *Picea*, and *Pinus* spp.). The PAC is currently comprised of 10 species, collectively the most studied of the so-called dark septate endophytes (DSE), an assemblage of distantly related fungi (also including species of e.g. *Cadophora*, *Leptodontidium*,

Microdochium, *Periconia*, *Trichocladium*) detected in roots of over 600 plant species species (Jumpponen and Trappe 1998, Knapp et al. 2012). Fungi grouping with the PAC are often among the most abundant recovered from root systems using culture-dependent or culture-independent sampling methods (Blaalid et al. 2012, Štursová et al. 2014, Bruzone et al. 2015, Li et al. 2015, Pickles et al. 2015). The relationship between PAC species and their hosts is complex, with interactions ranging from pathogenic (Wilcox and Wang 1987, Stoyke and Currah 1993, Tellenbach et al. 2011) to neutral or mutualistic (Fernando and Currah 1996, Vohník et al. 2005, Peterson et al. 2008, Newsham 2011, Tellenbach and Sieber 2012).

While most *Phialocephala*–host interaction studies focused on host growth and biomass, there is emerging evidence suggesting *Phialocephala* species may benefit the host through other complex interactions resulting in potentially beneficial outcomes, by production of antifeedant or antipathogenic secondary metabolites or other mechanisms (Tellenbach and Sieber 2012, Tellenbach et al. 2012, Terhonen et al. 2014). For example, a strain of *Ph. scopiformis* isolated as an endophyte from *Picea glauca* needles produces rugulosin, an insecticidal secondary metabolite with a reported dose-dependent weight reduction in eastern spruce budworm (*Choristoneura fumiferana*) (Miller et al. 2009). Miller (2011) suggested that the reduced growth rate of budworms could lead to longer exposure to adverse environmental and biotic factors, perhaps disrupting reproductive synchrony. Avirulent or weakly virulent PAC species may stimulate host defenses or suppress more severe root pathogens (e.g.: Tellenbach and Sieber 2012, Tellenbach et al. 2012) including more virulent PAC strains (Reininger et al.

2012) by space or nutrient competition or direct antagonism. Conversely, *Ph. bamuru* was recently described as the causal agent of fairway patch, a severe emerging disease of golf course turf in Australia that appears resistant to chemical control (Wong et al. 2015).

Despite the lack of comparable studies of other members of *Phialocephala* outside the PAC, they may be similarly abundant in poorly investigated niches, such as conifer needles, deciduous leaves, and living and dead wood. Several *Phialocephala* species (e.g. *Ph. glacialis*, *Ph. piceae*, *Ph. scopiformis*), and several other phylogenetically related but unnamed taxa with cystodendron-like asexual morphs, were repeatedly isolated from conifer needles, and may systemically infect their hosts in other organs, including roots (Hata and Futai 1995, 1996, Grünig et al. 2009). Such investigations indicate that leaf-colonization by *Phialocephala* species and allied taxa may be an important but little studied niche, particularly if similar secondary metabolites are expressed in host tissues, resulting in conditionally mutualistic benefits, as seen in root endophyte studies.

A major obstacle to understanding the niches and functions of *Phialocephala* species is that morphological approaches based on phialidic asexual morphs and nuclear internal transcribed spacer rDNA (ITS) sequences do not allow accurate identification or distinguishing of taxa. Identification of *Phialocephala* isolates using a morphotaxonomic approach is problematic because cultures are often apparently sterile and morphological variation may be insufficient for species delineation, especially among

closely related species (Grünig et al. 2008a). Sporulation in recalcitrant cultures has been induced by long-term incubation at low temperatures (Table 1.1), although sporulation in some species has never been observed (Grünig and Sieber 2005, Münzenberger et al. 2009). Morphological characters associated with conidiophores are taxonomically informative to an extent, but Grünig et al. (2008a) noted high intraspecific variation in asexual reproductive structures of PAC species. Molecular phylogenetic approaches also face many issues, reflecting difficulties in differentiating morphologically similar taxa that appear to be phylogenetically distinct. For example, Harney et al. (1997) identified a clade of three *Ph. dimorphospora* groups based on ITS sequences and rDNA restriction enzyme patterns, while Menkis et al. (2004) isolated eight *Ph. dimorphospora* ITS haplotypes from decaying *P. abies* stumps and boles. Phylogenetically differentiating closely related taxa using the ITS alone is questionable, as is evident with the *Ph. fortinii* s.l. complex, which Grünig et al. (2008a) determined to consist of at least six reproductively isolated species using a multi-locus approach using two coding (genes for β -tubulin and translation elongation factor 1- α) and three non-coding DNA loci (pPF-018, pPF-061, and pPF-076). However, ITS sequences appear to differentiate other described *Phialocephala* species.

Molecular evidence has repeatedly indicated a close relationship among named *Phialocephala* species and some species of *Mollisia*, a teleomorph genus associated with phialocephala-like asexual states (Vrålstad et al. 2002, Wilson et al. 2004, Menkis et al. 2005, Zijlstra et al. 2005, Wu and Guo 2008, Grünig et al. 2009, Day et al. 2012). In its current taxonomic state, *Mollisia* is an unwieldy para- and polyphyletic genus requiring

significant taxonomic revision, with at least 230 described species that are difficult or impossible to differentiate morphologically (Greenleaf and Korf 1980). Currently, few *Mollisia* sequences identified to species rank are accessioned in GenBank, and the difficulties associated with identifying species based on apothecia morphology calls these identifications into doubt. For example, sequences attributed to *M. cinerea* currently in GenBank probably represent several distinct and distantly related species based on their dissimilar ITS sequences. Genetic and morphological links with several other teleomorph genera have also been indicated, including *Phaeomollisia* (Johnston et al. 2014) and *Vibrissea* (Hamad and Webster 1988, Wang et al. 2006b, Grünig et al. 2009). Consequently, mollisoid apothecia may be candidate sexual states for some endophytes known only by their asexual morphs or sterile cultures.

Another important issue is that knowledge of *Phialocephala* spp. in the environment is limited to their endophytic or saprotrophic life history stages, ignoring their broader ecology. This at least partly reflects the lack of data on life cycles and dispersal mechanisms (e.g.: Grünig et al. 2008b, Zaffarano et al. 2010). Despite genetic and morphological data suggestive of the existence of sexual states, named *Phialocephala* spp. are currently unconnected with sexual states, probably because most studies include only cultures or environmental DNA sequences. Such links could perhaps be most readily investigated by combined studies involving isolation of endophytes, morphological characterization of the resulting cultures, and DNA sequencing of field-collected apothecia from the same forest, niche, biome or environment.

In this study, a survey of endophytes associated with conifers in Eastern Canada, with a focus on *Picea rubens*, was conducted to provide baseline biodiversity data and to identify sterile cultures of unidentifiable species by making connections with sporulating field specimens. Based on ITS sequences and morphology, many strains of described and unidentified *Phialocephala* spp. were isolated as endophytes of healthy needles. Endophytes identified as *Phialocephala* spp. were of particular interest because of their potential as a forest management tool against economically important forest pests (Miller et al. 2008). A combined lab and field-based approach facilitated the discovery of the sexual states of several described *Phialocephala* species, which were confirmed using DNA phylogenetic analyses of the ITS barcode sequences and the gene for the *largest subunit of RNA polymerase II (RPB1)*. Detailed morphological observations of these undescribed sexual morphs and their conidial states are provided.

MATERIALS AND METHODS

Sampling and isolation of fungi

Specimens were collected at several sites in New Brunswick, Canada and two sites in Aylmer, Quebec, Canada. Branches collected from understory and overstory conifers were stored in plastic bags at 4 C and subsequent isolations from conidia or ascospores occurred within 24 h or up to 10 d following collection. For endophyte isolations, needles were removed using forceps and surface sterilized by serial passage in 70% ethanol (1 min), 3% NaClO (7.5 min), and 70% ethanol (30 s). Needles then were rinsed in sterile distilled H₂O, blotted on sterile Kimwipes (Kimberly Clark, Mississauga,

Canada) and cut in half longitudinally. Needle segments were placed on 2% malt extract agar (MEA; 20 g Bacto malt extract, Difco Laboratories, Sparks, Maryland; 15 g agar, EMD Chemicals Inc., NJ, U.S.A.; 1 L distilled water) in 9 cm Petri dishes and incubated in the dark at 16 °C. Mycelia emerging from needle tissue were excised axenically and subcultured on 6 cm Petri dishes containing MEA.

Isolations from apothecial specimens were made by suspending sporulating ascospores from the lid of a Petri dish using petroleum jelly or drops of water, allowing ascospores to eject onto MEA. When possible, monospore cultures were obtained from individual ascospores transferred to 6 cm Petri dishes containing MEA. All cultures were incubated in the dark at 16 °C. Attempts to induce sporulation in culture involved prolonged incubation in the light or dark at 5, 10, and 15 °C for up to 18 mo and inoculation of isolates on various media; corn meal agar (CMA; Acumedia Manufacturers Inc., Lansing, MI, U.S.A.), oatmeal agar (OA; Crous et al. 2009b), oatmeal tomato paste agar (OA amended with 10 g tomato paste, 1 g MgSO₄ · 7H₂O, 1 g KH₂PO₄, 1 g NaNO₃), pine needle potato agar (PNPA; Su et al. 2012), and MEA and 1.5% water agar (WA, with 1 mL trace metal solution; Crous et al. 2009) with or without the addition of sterile *Picea* needles on the agar surface.

Morphological studies

Vertical sections of fresh apothecia were cut by hand and mounted in either water, 85% lactic acid, Melzer's reagent, 5% KOH, or Lugol's solution, with or without 5% KOH pretreatment. Colony colors were described using the alphanumeric codes of

Kornerup and Wanscher (1978). Microscopic measurements were taken from material mounted in water and are presented as ranges calculated from the mean \pm standard deviation of each measured value, with outliers in brackets. Observations were made using an Olympus BX50 light microscope and micrographs were captured using an Evolution MP Color Camera (Media Cybernetics, Silver Spring, CA, U.S.A.) and Image-Pro Plus v6.0 (Media Cybernetics) or InfinityX-32 camera (Lumenera Corp., Ottawa, ON, Canada) and Infinity Analyze v6.5.2 (Lumenera Corp.) software. Colony macrophotographs were captured with a Nikon Coolpix P5000 (Nikon Inc., Tokyo, Japan) and photographic plates were assembled using Adobe Photoshop v5.5 (Adobe Systems, San Jose, CA, U.S.A.).

To assess germination, conidia from two *Ph. dimorphospora* isolates (DAOMC 87232, DAOMC 250111) were collected using a fine tungsten inoculation loop, suspended in sterile Millipore water, and streaked onto plates containing MEA or CMA and incubated in the dark at 5, 10, 15, and 20 °C. Each treatment was conducted in triplicate and germination of conidia assessed visually every 24 h for 7 d.

Phylogenetic studies

Total genomic DNA was extracted from 4–12-wk old cultures or dried cultures (herbarium specimens) using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The primers ITS1 and ITS4 (White et al. 1990) or ITS4A and ITS5 (Larena et al. 1999) were used to amplify and sequence the ITS

region. The *largest subunit of RNA polymerase II (RPB1)* was amplified and sequenced using RPB1-Af and RPB1-6Rlasc 6Rlasc (Stiller and Hall 1997, Hofstetter et al. 2007). DNA was amplified using a PCR master mix consisting of 0.5 μ L 2 μ M dNTPs, 0.04 μ L 20 μ M forward primer, 0.04 μ L 20 μ M reverse primer, 1 μ L 10 \times Titanium *Taq* buffer (Clontech, Mountain View, CA, U.S.A.), 0.1 μ L 50 \times Titanium *Taq* enzyme (Clontech), and 1 μ L of DNA template, and 7.32 μ L sterile Milli-Q water (Millipore, Bedford, MA, U.S.A.) per reaction. All loci were amplified using the following PCR profile: 95 $^{\circ}$ C for 3 min, then 35 cycles at 95 $^{\circ}$ C for 1 min, 56 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 1.5 min, followed by a final extension at 72 $^{\circ}$ C for 10 min. PCR products were verified by agarose gel electrophoresis and sequenced with Big Dye Terminator (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence contigs were assembled and trimmed using Geneious R6 v6.1.8 (Biomatters, Auckland, New Zealand). The sequence of each locus was aligned using MAFFT v7 (Kato et al. 2005) and the resulting alignments trimmed and manually checked using BioEdit v7.2.5 (Hall 1999). All sequences used in this study are accessioned in GenBank (Supplementary Table 1.1) and the alignments and phylogenetic trees in TreeBASE (study no. 17404).

Phylogenetic analyses of single gene data sets were performed with Bayesian inference. The most suitable sequence evolution models for each gene (SYM+I+G for ITS, SYM+G for *RPB1*) were determined based on the optimal Akaike information criterion scores in MrModeltest v2.2.6 (Nylander 2004)). Bayesian analysis was performed with MrBayes v3.2 (Ronquist et al. 2012) with *Loramycetes macrosporus* as the

outgroup. For each locus, three independent Markov Chain Monte Carlo (MCMC) runs were run simultaneously for 3×10^6 generations (standard deviation of split frequencies <0.01), sampling every 500 generations. The first 25% of trees were discarded as burn-in and the remaining 6746 trees were kept and combined into one consensus tree with 50% majority rule consensus. Consensus trees were imported into FigTree v1.4.2 (Rambaut 2014) and exported as SVG vector graphics for assembly in Adobe Illustrator v10 (Adobe System, San Jose, CA, U.S.A.).

RESULTS

Isolations from surface-sterilized needle segments yielded 1010 isolates, including 79 isolates of *Phialocephala* spp. based on initial identifications using ITS barcodes. *Phialocephala scopiformis* was the most abundant species, represented by 53 isolates, with *Ph. piceae* the second most abundant, represented by 14 isolates. Two isolates belonging to a well-defined clade including *Ph. dimorphospora* (henceforth referred to as the *Ph. dimorphospora* clade) were recovered (NB-249-2D, NB-105-2B) and the remaining 10 isolates represented seven unnamed ITS phylotypes phylogenetically related to *Phialocephala* s.l., to be considered in subsequent publications.

A total of 128 mollisioid apothecial specimens were collected from a variety of woody substrates in the same forest stands sampled for endophytes. Colony morphologies of isolates resulting from ascospore isolations generally resembled those of *Phialocephala* endophyte isolates. Comparison of ITS sequences of *Phialocephala*

endophytes and isolates derived from mollisoid ascomata indicated the discovery of apothecia corresponding to *Ph. scopiformis*, *Ph. nodosa* sp. nov., and *Ph. piceae* (Fig. 1.1). Connections between endophyte strains and corresponding apothecia field collections were further confirmed with *RPB1* sequences (Fig. 1.2).

Apothecia for most species could not be unambiguously differentiated in the field, although apothecia of *Ph. scopiformis* had a characteristic blue hymenium color. Several species in the *Ph. dimorphospora* clade were readily distinguished based on the morphology of asexual states on MEA, while others were clearly delineated by ITS sequences. These species include *Ph. nodosa*, characterized by darkly pigmented microsclerotia consisting of irregularly coiled 1–2-septate cells; *Ph. catenospora*, which produces two synanamorphs consisting of typical penicillate conidiophores and pigmented 1–4-septate diplococcium-like conidia in simple or branched chains; and *Ph. aylmerensis*, which produces two types of conidiophores producing minute conidia in slimy heads, hyaline reduced conidiophores and pigmented 1–2 branching conidiophores. One isolate was not observed to form a conidial state, but was conspecific with the synnematosous fungus *Paradidymobotryum oblongum* (99% identity based on ITS sequences; K. Seifert, unpubl.), and is therefore named *Ph. oblonga* due to priority. Conidial states were not observed for *Ph. mallochii*.

The type specimen of *Ph. dimorphospora* [DAOM 71465(c)] consists of a herbarium sheet with four herbarium packets. The holotype is comprised of four small pieces of *Ulmus* sp. (probably *U. americana*) wood with sparse *Ph. dimorphospora*

conidiophores. Two packets contain slides and micrographs of the type specimen and one packet contains five dried ex-type cultures. Living ex-type cultures of *Ph. dimorphospora* no longer exist (C. Babcock, pers. comm.) and attempts to sequence ITS and *RPB1* from the dried cultures associated with the type specimen of *Ph. dimorphospora* failed. There were insufficient quantities of DNA or the resulting sequences were of *Malassezia globosa*, presumably a contaminant on the herbarium specimens. A *Ph. dimorphospora* culture isolated from paper mill slime in New Brunswick, Canada (DAOMC 87232 = CBS 300.62) and authenticated by W.B. Kendrick is selected below as an epitype. This culture is morphologically consistent with the type specimen and produces abundant conidiophores on MEA.

In 2014, a *Ph. dimorphospora* specimen (DAOMC 250111) was collected from decaying hardwood (*Populus* sp.) ca. 30 km northeast of the type locale of *Ph. dimorphospora*. Based on morphology and ITS and *RPB1* sequences, this specimen is conspecific with the selected *Ph. dimorphospora* epitype (DAOMC 87232). Conidia from DAOMC 87232 and DAOMC 250111 streaked on CMA and MEA and incubated at 10, 15, and 20 °C germinated within 72 h, confirming their function as propagules and not exclusively as spermatia.

Phialocephala scopiformis, *Ph. piceae*, and members of the *Ph. dimorphospora* clade were consistently delineated in both ITS and *RPB1* phylogenies. The *Ph. dimorphospora* clade formed a strongly supported [posterior probability (P.P.) = 1] polytomous clade

containing *Ph. aylmerensis*, *Ph. catenospora*, *Ph. dimorphospora*, *Ph. lagerbergii*, *Ph. mallochii*, *Ph. nodosa*, *Ph. oblonga*, *Ph. repens*, and *Mollisia heterosperma*.

TAXONOMY

All species described below, except *Phialocephala scopiformis* and *Ph. piceae*, are part of what we interpret as the core clade of *Phialocephala* s.s. following single name nomenclature (Hawksworth et al. 2011), which includes species with different combinations of mollisoid sexual states, phialidic asexual states that may or may not have penicillate conidiophores, companion asexual states with acropetal chains of septate conidia (here called diplococcium-like), similar diplococcium-like asexual states producing synnemata, or microsclerotia. *Phialocephala scopiformis* and *Ph. piceae* are treated as *Phialocephala* s.l. and their final classification requires resolution of other taxonomic issues not addressed here. *Mollisia heterosperma* is treated as a member of the core *Phialocephala* s.s. clade, but its reclassification awaits a broader treatment of *Mollisia* and the phylogenetic placement of an epitype for *M. cinerea*. We (i) propose an epitype specimen for *Ph. dimorphospora*, the type species of the genus; (ii) add descriptions of sexual morphs to the previously asexual concept of *Ph. scopiformis*; (iii) make the first report of *Ph. piceae* apothecia collected in the field and expand on the protologue; (iv) describe the new species *Ph. nodosa* with apothecia and a microsclerotial asexual state, (v) describe *Ph. catenospora* with apothecia, a penicillate anamorph and diplococcium-like anamorph, (vi) describe *Ph. mallochii* with apothecia only, and (vii) describe *Ph. aylmerensis* with apothecia and a phialidic asexual state, and

make a new combination *Ph. oblonga* for a species with apothecia and a synnematosus diplococcium-like asexual state previously described as the type of the hyphomycete genus *Paradidymobotryum*. For all descriptions, details of additional specimens, cultures and GenBank accession numbers for DNA sequences are provided in Supplementary Table 1.1.

Phialocephala dimorphospora W.B. Kendrick, Can. J. Bot. 39: 1080. 1961.

Typification: Canada, Ontario, Manitowick, on rotten wood of *Ulmus* sp., 4 Nov 1960, S.J. Hughes (holotype, DAOM 71465(c)). NEW BRUNSWICK: a dried culture originally isolated from slime in pulp mill, 1961, W.B. Kendrick (epitype designated here, DAOM 574894. Ex-epitype culture DAOMC 87232 (= CBS 300.61 = CMW 665 = UPSC 2186).

Phialocephala scopiformis T. Kowalski & R.D. Kehr, Can. J. Bot. 73: 27. 1995.

Figs. 1.3, 1.4

Colonies 19–21 mm diam after 14 d in the dark at 20 °C on MEA; flat to slightly convex in center, sparse greyish brown (8F3) funiculate hyphae in centre, margin diffuse, flat, wide, yellowish brown (5E5) to white; surface greyish brown to dark brown (8F3–8F4) with wide (2–3 mm), yellowish brown (5E5) outer concentric circle; reverse dark brown (7F4) with yellowish brown to white margin (5E5). Exudates and soluble pigments absent in younger cultures, older (>4 mo) cultures exuding small yellowish orange (4B4) droplets on colony surface and surrounding agar turning greyish yellow

(3B5), yellowish orange (4B4) plate-like crystals sometimes present on surface of colony or below agar surface.

Apothecia scattered to gregarious or caespitose; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to concave at maturity; outline entire, sometimes sinuate; greyish blue (21E7), outer surface slightly darker; 0.3–1 mm diam, 0.2–0.3 mm high; margin sometimes paler because of refractive contents in subhyaline marginal cells, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 25–105 μm thick near base, 15–40 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (12–)12.5–18(–21) \times (8–)8.5–11.5(–12) μm ; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to elongated clavate cells with +/- thin walls, 8–12(–15) \times (6–)6.5–8(–9) μm ; marginal cells organized in distinctive parallel rows, obovoid to clavate or spathulate, 9–12(–12.5) long, maximum width towards apex 4–5.5(–7) μm , minimum width at base 2–3 μm ; brownish orange to brown (5D4–6E4) around margin and becoming dark brown (5F8) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to dark green (1E6–27F5) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, sometimes gelatinized, 2.5–3.5(–4) μm , thick-walled (<0.5–1 μm), dark brown (5F8). Medullary excipulum hyaline, *textura intricata*, 20–35 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–3.5(–4) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction: negative. Asci arising from croziers, cylindrical-clavate, 8-spored, 35–45(–55) \times 3–5.5 μm , pars sporifera 20–25 μm , pore amyloid in

Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (6.5–)8–9.5(–11) × (2–)2.5–3 μm, ellipsoidal to oblong-ellipsoidal or fusoid-clavate, apices rounded with one apex often conspicuously acute, 0(–1) septate, thin-walled, frequently guttulate with 2–3(–4) large guttules (1–2.5 μm diam) or several small guttules (<0.5 μm diam) aggregated at both poles.

See Kowalski and Kehr (1995) for description of the *Ph. scopiformis* asexual state.

Cardinal temperatures: Range 5–30 °C, optimum 20–25 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Associated with decaying wood and living foliage and branches of conifers, including *Picea abies*, *P. glauca*, *P. mariana*, *P. rubens*, and *Pinus strobus*.

Distribution: Canada (New Brunswick), Germany, United Kingdom.

Additional specimens and cultures examined: CBS 468.94, see Supplementary Table 1.1.

Notes: *Phialocephala scopiformis* is frequently isolated as an endophyte of living *Picea* spp. and *Pinus strobus* needles. In this study, apothecia were only found on decaying *Picea rubens* branches. Apothecia are distinguished by marginal cells that are organized in distinctive parallel rows, a thin medullary excipulum, heavily melanised ectal excipulum, and occasionally sinuate outline. The characteristic blue hymenium color is observable in the field.

Phialocephala piceae (T.N. Sieber & C.R. Grünig) Rossman, IMA Fungus 5: 104. 2014.

Figs. 1.3, 1.5

≡ *Phaeomollisia piceae* T.N. Sieber & C.R. Grünig, Mycol. Res. 113(2): 215. 2009

(Basionym)

Colonies 32–36 mm diam after 14 d in the dark at 20 °C on MEA; flat to slightly convex in center, woolly olive brown (4E3) aerial mycelia in center, margin diffuse, flat, and white; surface olive brown (4E5–4F4); reverse olive grey (3F2). Exudates and soluble pigments absent.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, becoming plain to slightly concave at maturity; outline entire; greyish brown to yellowish brown (5D3–5D5), outer surface slightly darker; 0.5–2 mm diam, 0.2–0.4 mm high; margin lighter color, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 50–140 µm thick near base, 15–40 µm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, 13–16.5(–19) × (8–)9.5–12(–12.5) µm; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to elongate cylindrical to slightly clavate cells with +/- thin walls, 6–8.5(–10) × (5–)5.5–7.5(–9) µm; marginal cells cylindrical to clavate, (9–)11–18(–20) µm long, maximum width towards apex 5–8 µm, minimum width at base 3–5 µm; hyaline to greyish yellow (4B4) around margin and becoming light brown (6D7) toward base, not gelatinized, crystals or exudates absent gelatinized; tissue becoming olive (3E5) when mounted in 5% KOH. Subicular hyphae

sparse, sometimes gelatinized, 2–3(–3.5) μm , thick-walled (0.5–1 μm), dark brown (5F8). Medullary excipulum hyaline, textura intricata, 32–48(–55) μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 2–2.5(–3) μm wide, containing large highly refractive vacuole bodies; not exceeding the length of mature asci. KOH reaction: strong, paraphyses turning yellow (3A6), visible with unaided eye. Asci arising from croziers, cylindrical-clavate, 8-spored, (33–)37–49(–53) \times 4–7 μm , pars sporifera 20–30 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (7.5–)9–12(–15) \times (2–)2.5–3(–3.5) μm , ellipsoidal to ellipsoidal-fusiform, apices rounded, 0(–1) septate, thin-walled, several <0.5–1(–2) μm diam guttules usually aggregated at both poles.

See Grünig et al. (2009) for description of the phialocephala-like and cadophora-like asexual states of *Ph. piceae*.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea abies*, *P. glauca*, *P. mariana*, *P. rubens*, and *Pinus strobus* needles and decomposing branches or wood of *Acer saccharum*.

Distribution: Canada (New Brunswick), Lithuania, Sweden, Switzerland.

Additional specimens and cultures examined: see Supplementary Table 1.1.

Notes: *Phialocephala.piceae* apothecia are distinguished by their yellowish brown color and by often being erumpent from bark of host branches (*Acer saccharum*).

Phialocephala nodosa J.B. Tanney & B. Douglas, sp. nov. Figs. 1.3, 1.6O–S, 1.7

MycoBank MB811718

Typification. Canada, New Brunswick, Alma, Fundy National Park, Maple Grove trail, 45.58178 -64.98633, from decaying *Acer saccharum* branch, 17 Jul 2014, J.B.

Tanney NB-475 (**holotype** DAOM 628553). Ex-type culture DAOMC 250115.

Etymology: *nodosa* (Latin), knotty, referring to the appearance of microsclerotia produced in culture.

Colonies 20–22 mm diam after 14 d in the dark at 20 °C on MEA; flat to convex in center, woolly olive grey (3E2) aerial mycelia in center, margin diffuse, flat, and white; surface brownish grey to olive brown (4F2–4F5); reverse olive grey (3E2); shiny black microsclerotia sparsely to moderately abundant in aerial mycelia. Exudates and soluble pigments absent. Mycelium consisting of subhyaline to dematiaceous, smooth, septate, branched, hyphae 2–3.5 µm diam, sometimes covered with gelatinous sheaths 1–3.5 µm diam. Helicoid initials proliferate into dense microsclerotia comprised of darkly pigmented, moniliform cells, 3–6 × 1.5–3.5 µm. Microsclerotia abundant in aerial mycelia and later forming crust on colony surface on MEA, OA, and CMA.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to concave at maturity; outline entire; orange grey

to greyish brown (5B2–5E3), outer surface darker; 0.6–2 mm diam, 0.2–0.4 mm high; margin frequently paler color, smooth. Ectal excipulum at base and mid flanks textura globulosa to angularis, 32–125 μm thick near base, 27–36 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (14–)16–19.5(–22) \times (8–)9–12 μm ; at upper flank and margin textura angularis to prismatica, composed of globose to elongated clavate cells or cylindrical with +/- thin walls, 7–9.5(–11.5) \times (5.5–)6–7 μm ; marginal cells cylindrical to obovoid or clavate, (–8)11–19(–20.5) long, maximum width towards apex 6–7.5(–8) μm , minimum width at base (4.5–)5–6(–6.5) μm ; brownish orange to brown (5D4–6E4) around margin and becoming dark brown (6F7) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to dark green (1E6–27F5) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, sometimes gelatinized with conspicuously thick (2–3.5 μm) layer, 2.5–3.5(–4) μm , thick-walled (0.5–1 μm), dark brown (5F8). Medullary excipulum hyaline, textura intricata, 28–36(–45) μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–3.5(–4) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction: negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (49–)51.5–62(–65) \times 5–6.5 μm , pars sporifera 19–30 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (7–)8–10.5(–12) \times (2–)2.5–3(–4) μm , ellipsoidal-fusiform to oblong, apices rounded, aseptate, thin-walled, frequently guttulate with 3–7 guttules (<0.5–1 μm) aggregated towards both poles.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Endophyte of healthy *Picea mariana* and *Pinus strobus* needles and decomposing branches or wood of *Acer rubrum*, *A. saccharum*, *Betula cordifolia*, and *B. papyrifera*.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: see Supplementary Table 2.

Notes: *Phialocephala nodosa* is distinguished by the abundant microsclerotia produced in aerial mycelium, later forming a crust on the colony surface.

Phialocephala catenospora J.B. Tanney & B. Douglas, sp. nov. Figs. 1.3, 1.6A, H–N, 1.8

MycoBank MB811719

Typification. Canada, New Brunswick, Charlotte County, Little Lepreau, 45.136830 -66.481550, from decaying *Betula papyrifera* branch, 13 Jul 2014, J.B. Tanney NB-432 (**holotype** DAOM 628547). Ex-type culture DAOMC 250108.

Etymology: *catena* (Latin), chained together, to describe the conidial chains.

Colonies 25–30 mm diam after 14 d in the dark at 20 °C on MEA; flat with fascicular aerial hyphae aggregated in centre; margin wide, diffuse, and white; surface greyish brown to olive brown (5F3–4F8); reverse brownish grey (4E2). Exudates and soluble pigments absent. *Mycelium* consisting of subhyaline to dematiaceous, smooth,

septate, branched hyphae, 2–3.5 μm diam, sometimes covered with gelatinous sheaths 1–3.5 μm diam. Diplococcium-like and *Phialocephala* synanamorphs both observed.

Diplococcium-like synanamorph: Conidiophores macronematous, erect, brown, smooth, cylindrical, thick-walled, unbranched to once or twice branched, length indeterminate, 3.5–5 μm wide, pluriseptate, occurring singly or caespitose; conidiogenous cells monoblastic or polyblastic, integrated, terminal or intercalary, extensions sympodial, with 1–2 conidiogenous loci, subdenticulate. Conidia (10–)12–22.5(–28.5) \times (4.5–)5–6 μm , 1–3(–5)-septate, dry, catenate, acropetal, in unbranched or branched chains from ramoconidia, subcylindrical, rounded to subtruncate base, sometimes slightly constricted at septa, brown, often versicolorous with one cell (usually basal) paler, smooth, basal cell with a lighter, refractive, flattened to protruding hilum, 1 μm wide;

Phialocephala synanamorph: Conidiophores micronematous to macronematous, erect, subhyaline to pale brown, cylindrical, thin-walled, solitary to divaricate with 2–4 metulae, metulae smooth, subhyaline to pale brown, thin-walled, (5–)6–8(–9) \times (2.5–)3–4 μm , 2–3(–4) conidiogenous cells arising from metula, conidiophores becoming more septate, melanised, and densely branched with maturity. Conidiogenous cells phialidic, terminal or intercalary, (7–)9–13 \times (2.5–)3–3.5 μm , collarettes cylindrical to flaring, 2–3(–4) \times 2–2.5 μm , hyaline to pale brown. Mature conidiogenous cells sometimes developing into diplococcium-like synanamorph. Conidia 2–3.5(–4.5) \times (1.5–)2 μm , aseptate, hyaline, subglobose to obovoid or oblong, occurring in slimy heads.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to shallowly concave at maturity; outline entire;

hymenial surface smooth to finely pruinose because of adherence of ejected ascospores, bluish grey to greyish blue (20C3–21C4), outer surface slightly darker; 0.4–1.2 mm diam, 0.2–0.3 mm high; margin not differentiated, smooth. Ectal excipulum at base and mid flanks textura globulosa to angularis, 40–110 μm thick near base, 15–30 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (11–)12.5–16(–18) \times (6–)8–10.5(–11) μm ; at upper flank and margin textura angularis to prismatica, composed of globose to elongated clavate cells with +/- thin walls, 6–8.5(–10) \times (5–)5.5–7.5(–9) μm ; marginal cells cylindrical-clavate to obovoid, (9–)11–18(–25) μm long, maximum width towards apex 2.5–5 μm , minimum width at base 2–3 μm ; hyaline to brownish orange (5C3) around margin and becoming dark brown (5F8) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to olive grey (3F4 to 3F2) when mounted in 5% KOH. Subicular hyphae sparse to abundant, sometimes thinly gelatinized, 2–3.5(–4) μm , thick-walled (0.3–1 μm), dark brown (5F8). Medullary excipulum hyaline, textura intricata, 30–60 μm thick.

Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, (2.5–)3–3.5(–4) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci.

KOH reaction: negative, rarely weak pale yellow. Asci arising from croziers, cylindrical-clavate, 8-spored, biseriate, (53–)54–67(–69.5) \times 6–8. μm , pars sporifera 21–26 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores (6.5–)7–8.5(–10) \times (2–)2.5–3 μm , oblong to fusiform or clavate, straight to allantoid or slightly

sigmoid, aseptate, thin-walled, frequently guttulate with 1–6 guttules (0.5 µm diam) aggregated towards both poles.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum slightly <5 °C, maximum slightly >35 °C.

Host range: Associated with decomposing wood of *Acer rubrum*, *Betula alleghaniensis*, and *B. papyrifera*.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: see Supplementary Table 1.1.

Notes: *Phialocephala catenospora* is distinguished by the diplococcium-like asexual state observed in culture.

Phialocephala mallochii J.B. Tanney & B. Douglas, sp. nov. Figs. 1.3, 1.9

Mycobank MB811720

Typification. Canada, New Brunswick, Charlotte County, Little Lepreau, 45.140744 -66.479359, from decaying *Alnus viridis* stem, 12 Jul 2014, *B. Malloch* NB-430, (**holotype** DAOM 628552). Ex-type culture DAOMC 250113.

Etymology: Named for its initial collector, Bruce Malloch.

Colonies 20–23 mm diam after 14 d in the dark at 20 °C on MEA; convex, densely woolly, olive brown (4E3) aerial mycelia; diffuse white margin; surface olive (4E5–4F4);

reverse dark grey to brownish grey (1F1–4F2). Exudates and soluble pigments absent. Asexual state not observed.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to concave at maturity; outline entire; bluish grey (23E3), outer surface darker; 0.5–2 mm diam, 0.2–0.4 mm high; margin frequently lighter color because of cells containing refractive vacuole bodies, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 35–128 μm thick near base, 23–38 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, 7.5–11(–15) \times (5–)6–7.5(–9) μm ; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to elongated clavate cells or cylindrical with +/- thin walls, 10–16(–21) \times (5.5–)7–9(–10) μm ; marginal cells obovoid to clavate or spatulate, 9–16(–17) μm long, maximum width towards apex 5.5–7(–7.5) μm , minimum width at base 2–3.5 μm , frequently containing refractive vacuole bodies; brownish orange to brown (5D4–6E4) around margin and becoming dark brown (6F7) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to dark green (1E6–27F5) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, sometimes gelatinized, 2–3(–4) μm , thick-walled (0.4–1 μm), dark brown (5F8). Medullary excipulum hyaline, *textura intricata*, 23–43 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–4(–4.5) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction: negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (34–)37–46(–52) \times 3.5–5(–6.5) μm , pars sporifera 18–26 μm , pore amyloid in Melzer's reagent or

Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (7–)8–11.5(–14) × (2.5–)3–4(–4.5) μm, ellipsoidal to fusoid-clavate or oblong, apices rounded, 0–1(–3) septate, thin-walled, frequently guttulate with many 0.5–2 μm diam guttules aggregated towards both poles.

Cardinal temperatures: Range 5–30 °C, optimum 25 °C, minimum <5 °C, maximum slightly >30 °C.

Host range: Associated with decomposing wood of *Acer saccharum* and *Alnus viridis*.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: see Supplementary Table 1.1.

Notes: *Phialocephala mallochii* is distinguished by ascospores containing up to 3 septa.

Phialocephala oblonga (C.J.K. Wang & B. Sutton) J.B. Tanney, Seifert & B. Douglas,

comb. nov. Figs. 1.3, 1.6B–D, 1.10

Mycobank MB811722

≡ *Paradidymobotryum oblongum* C.J.K. Wang & B. Sutton, Mycologia 76: 572. 1984

(Basionym).

Colonies 24–27 mm diam after 14 d in the dark at 20 °C on MEA; convex, abundant woolly, olive grey (3E2) aerial mycelia; diffuse white margin; surface brownish grey to olive brown (4F2–4F5); reverse dark grey to brownish grey (1F1–4E2). Exudates and soluble pigments absent.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to concave at maturity; outline entire; greyish blue to deep blue (21D6–22E8), outer surface darker; 1.5–2.5 mm diam, 0.2–0.3 mm high; margin frequently lighter color due to cells containing refractive vacuole bodies, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 85–130 μm thick near base, 21–53 μm thick towards margin; at base and lower flank composed of globose to isodiametric cells with thin to slightly thickened walls, (17.5–)18.5–24(–27.5) \times (8–)10.5–15.5(–17) μm ; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to elongated clavate cells or cylindrical with +/- thin walls, 9–11(–12) \times (5.5–)6–8(–9.5) μm ; marginal cells obovoid to clavate or spatulate, (9–)10.5–15.5(–15) long, maximum width towards apex (5.5–)6–8(–8.5) μm , minimum width at base (2.5–)3.5–5(–5.5) μm , frequently containing refractive vacuole bodies; brownish orange to brown (5D4–6E4) around margin and becoming dark brown (6F7) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to dark green (1E6–27F5) when mounted in 5% KOH. Subicular hyphae usually sparse, 2–3 μm , thick-walled (0.5 μm), dark brown (5F8). Medullary excipulum hyaline, *textura intricata*, 22–42 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 2.5–3.5 μm wide, containing large highly refractive vacuole bodies; not

exceeding mature asci. KOH reaction: negative. Asci cylindrical-clavate, 8-spored, (49–)58–76 × (4–)4.5–6(–7) μm, pars sporifera 20–25 μm, pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution; arising from croziers. Ascospores biseriate to obliquely uniseriate, (8–)9–11.5(–17) × (2.5–)3–3.5(–4) μm, elliptic-fusiform to oblong, sometimes appearing bent, apices rounded, aseptate, thin-walled, frequently guttulate with guttules 0.5–1 μm diam aggregated towards both poles and sometimes bearing two larger guttules 2–2.5 μm diam.

See Wang & Sutton (1984) for description of the asexual state (as *Paradidymobotryum oblongum*).

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum slightly <5 °C, maximum slightly >35 °C.

Host range: Associated with decomposing wood of *Acer saccharum*, *Betula alleghaniensis*, and *Ulmus americana*.

Distribution: Canada (New Brunswick, Ontario) and U.S.A. (New York).

Additional specimens and cultures examined: DAOMC 250119, see Supplementary Table 1.1.

Notes: *Phialocephala oblonga* is characterized by long ascospores and its synnematosus asexual state.

Phialocephala aylmerensis J.B. Tanney & B. Douglas, sp. nov. Figs. 1.3, 1.6T–X, 1.11

Mycobank MB811721

Typification. Canada, Quebec, Aylmer, Jardin Lavigne Park, 45.418969 - 75.834870, from decaying *Betula papyrifera* log, 16 Sep 2014, *J.B. Tanney & B. Tanney NB-543*, (**holotype** DAOM 628548). Ex-type culture DAOMC 250106.

Etymology: Named for the type locale, Aylmer, Quebec.

Colonies 22–27 mm diam after 14 d in the dark at 20 °C on MEA; flat, sparse fascicular olive grey (3E2) aerial mycelia mostly aggregated in center; diffuse white margin; surface brownish grey to olive brown (4F2–4F5); reverse dark grey to brownish grey (1F1–4F2). Exudates and soluble pigments absent. *Mycelium* consisting of subhyaline to dematiaceous, smooth, septate, branched, hyphae 2–3(–3.5) µm diam, sometimes covered with gelatinous sheath 1–3.5 µm diam. Conidiophores micronematous to macronematous, erect, pale to dark brown, smooth, cylindrical, thin- or thick-walled, unbranched or occasionally singly branched, up to 17.5 × 3.5 µm, pluriseptate. Conidiogenous cells phialidic, terminal, sometimes intercalary, ampuliform, (7–)8.5–13(–17) × (2–)3–4(–4.5) µm, collarettes cylindrical to flaring, 2.5–4(–5) × 2–2.5 µm, hyaline to pale brown.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plain to concave at maturity; outline entire to sinuate; pale grey (1B1) to bluish grey (23E3), outer surface darker; 1–3 mm diam, 0.2–0.4 mm

high; margin frequently lighter color because of cells containing refractive vacuole bodies, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 49–105 μm thick near base, 19–38 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, 14–19(–25) \times (7.5–)10–13(–14) μm ; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to elongated clavate cells or cylindrical with +/- thin walls, (14–)15–20(–23) \times (8–)11–13(–14) μm ; marginal cells cylindrical to obovoid to clavate or spathulate, (13–)14–18(–20.5) μm long, maximum width towards apex 5.5–7.5(–9) μm , minimum width at base 2–3.5 μm , frequently containing refractive vacuole bodies; hyaline to brownish grey (5D3) around margin and becoming greyish brown (6F3) toward base, not gelatinized, crystals or exudates absent; tissue becoming olive to dark green (1E6–27F5) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, 2–4(–4.5) μm , thick-walled (0.5–1 μm), dark brown (5F8). Medullary excipulum hyaline, *textura intricata*, 40–58 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 2.5–3(–4) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction: negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (50–)52–60(–62) \times 6–7 μm , pars sporifera 18–23 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, 7–9(–10.5) \times 3–3.5(–4) μm , oblong to fusiform or clavate, straight to allantoid, aseptate, thin-walled, frequently guttulate with 2–7 guttules (0.5–1 μm diam) aggregated towards both poles.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum slightly <5 °C, maximum slightly >35 °C.

Host range: Associated with decomposing wood of *Betula papyrifera*.

Distribution: Canada (Quebec).

Additional culture examined: see Supplementary Table 1.1.

Notes: *Phialocephala aylmerensis* is characterized by reduced phialides borne directly on hyphae or on short conidiophores.

DISCUSSION

The detection of *Phialocephala* sexual states and corresponding endophytic and anamorphic states within the same forest stands provides evidence of life cycles involving vegetative endophytic stages, saprotrophic stages with phialidic states on non-foliar substrates, and the formation of mollisoid sexual morphs enabling widespread ascospore dispersal. Although we isolated *Ph. nodosa*, *Ph. piceae*, and *Ph. scopiformis* as foliar endophytes of conifers, sexual states were not observed on fresh, senescent, or abscised needles. During the summer and fall seasons in the sampling areas, mollisoid sexual states of these species were commonly encountered on woody substrates ranging from intact fallen branches to old coarse woody debris, usually originating from angiosperm trees. These observations suggest the ability of those *Phialocephala* species to occupy several niches as either specialists or generalists, indicating complex and diverse life-history strategies.

Phialocephala piceae was described from a *Picea abies* needle endophyte isolate in Switzerland that produced apothecia with dark ascospores in vitro (Grünig et al. 2009). It was also detected in a stump of *Picea abies* and a dead stem of *Betula pendula* (Menkis et al. 2004). In this study, *Ph. piceae* was isolated as an endophyte from *Picea glauca*, *P. mariana*, *P. rubens*, and *Pinus strobus*, and its sexual state was collected only on fallen branches of *Acer saccharum*. Our specimen appears to be the first collection of *Ph. piceae* apothecia from nature. Pigmented ascospores were not observed in our collections, possibly a result of apothecial immaturity, cultural artifacts in the original study, or differences in geographic races. Some collections had apothecia erumpent through the bark of the host branch, a distinctive character that suggests its presence as an endophyte in woody tissue before branch drop.

Members of the *Ph. dimorphospora* clade have been most commonly isolated as DSE from coniferous tree roots or from decomposing wood (Menkis et al. 2004), although several other ITS sequences and distinct ITS phylotypes present in GenBank have been reported from other substrates (e.g. EU434847 *Ph. repens* from *Populus*, and JF340261 *Phialocephala* sp. M49 from *Alnus*). In this study, apothecia assignable to the *Ph. dimorphospora* clade were collected from deciduous wood (*Acer saccharum*, *Alnus viridis*, *Betula alleghaniensis*, *B. cordifolia*, *B. papyrifera*, and *Fagus grandifolia*) that was usually decorticated and in advanced states of decomposition. *Phialocephala dimorphospora* s.l. causes soft rot and significant weight loss in deciduous wood (Nilsson 1973, Morrell and Zabel 1985, Wang and Zabel 1990, Held 2013) and is implicated in wood staining (Kowalski 1991). Menkis et al. (2004) hypothesized that *Ph.*

dimorphospora may latently infect healthy trees, causing wood staining when the health of the host declines, and decomposing wood following host death. Latent infection of living branches may also play a role in natural pruning (Butin and Kowalski 1986, Kowalski and Kehr 1992, Barklund and Kowalski 1996, Kowalski and Zych 2002). Despite members of this clade being among the most commonly field-collected apothecia in this study, only two isolates of the *Ph. dimorphospora* clade were recovered as endophytes from *Pinus strobus* needles (all *Ph. nodosa*), suggesting needle colonization may be infrequent and opportunistic.

Menkis et al. (2004) isolated a cluster of eight *Ph. dimorphospora*-affiliated ITS-phylotypes from *P. abies* stumps and boles, which they classified as one species because of the lack of differentiating characters or available sequences of other *Phialocephala* spp. in this clade. Our results confirm that the *Ph. dimorphospora* clade is comprised of genetically distinct species, many of which produce distinctive asexual states. A well-defined *Ph. dimorphospora* was required to precisely delineate distinct species in this clade and establish *Phialocephala* s.s. as a generic concept. There is no existing ex-type culture and attempts to sequence DNA from dried cultures associated with the type specimen failed. We therefore selected an authenticated *Ph. dimorphospora* isolate (DAOMC 87232 = CBS 300.62) as epitype based on its congruency with the species concept. In the taxonomy section above, the core *Phialocephala* s.s. clade is defined to include *Ph. aylmerensis*, *Ph. catenospora*, *Ph. dimorphospora*, *Ph. lagerbergii*, *Ph. mallochii*, *Ph. nodosa*, *Ph. oblonga*, *Ph. repens*, and *Mollisia heterosperma*.

Our observations alter the monomorphic generic concept of *Phialocephala* to include sexual states and additional synanamorphs. Some species in the *Ph. dimorphospora* clade produced distinct conidial states previously attributable to other poorly sampled hyphomycete genera (e.g. *Bispora*, *Diplococcium*, *Paradidymobotryum*, *Septonema*). While such conidial states have not been formally associated with *Phialocephala*, *Diplococcium spicatum* (CBS162.47, CBS852.73), *Trimmatostroma betulinum* (CBS282.74), and *T. salicis* (CPC13571) sequences in GenBank are closely related to *Phialocephala* and *Mollisia* (Crous et al. 2007, Shenoy et al. 2010, Lin et al. 2011). *Trimmatostroma*, a phylogenetically heterogeneous asexual genus, is characterized by arthric chains of brown phragmo- or dictyoconidia while *Diplococcium*, also phylogenetically heterogeneous, is characterized by brown, didymo- or phragmoconidia in branched or unbranched acropetal chains from polytretic conidiogenous cells (Seifert et al. 2011). *Phialocephala catenospora* produced a diplococcium-like conidial state with acropetal chains of 1–4-septate, blastic conidia. This probably explains why accessioned sequences of fungi collected from wood and identified as *Trimmatostroma* or *Diplococcium* are sometimes associated in the phylogenetic and morphotaxonomic literature with *Phialocephala* or *Mollisia* states (Crous et al. 2007, Shenoy et al. 2010). Notably, Le Gal and Mangenot (1956) described a *Mollisia* sp. culture ("*Mollisia* sp. 1 from *Betula*") with two synanamorph that are very similar to those observed in our cultures of *Ph. nodosa*.

Phialocephala oblonga is based on *Paradidymobotryum oblongum*, the type species of a monotypic hyphomycete genus described from rotten *Ulmus americana*

wood collected in New York State (Wang and Sutton 1984). This connection was confirmed by comparing ITS and *RPB1* sequences of two cultures derived from single spore isolations of independently collected *Ph. oblonga* specimens. This synanamorph connection was unexpected because of the striking differences in morphology and conidiogenesis of *Pa. oblongum* and *Phialocephala*. However, there are morphological similarities between *Pa. oblongum* and the diplococcium-like synanamorph of *Ph. catenospora*.

Authentic cultures of *Mollisia heterosperma* (CBS 292.59), *Ph. lagerbergii* (ex-type culture; CBS 266.33), and *Ph. repens* (purported ex-type culture fide Grunig et al. 2009; MUCL1849) also belong to the *Ph. dimorphospora* clade. The known asexual state of *M. heterosperma* consists of penicillate conidiophores with deep collarettes bearing both subglobose and ovoid conidia, reminiscent of *Ph. dimorphospora*. Although morphologically similar, Kendrick (1963) distinguished *Ph. repens* from *Ph. dimorphospora* because the former species lacked the characteristic elongated primary conidium and consequently had shorter collarettes. Melin and Nannfeldt (1934) described *Ph. lagerbergii* from blue-stained *Pinus sylvestris* wood in Sweden. This species is characterized by ellipsoidal or reniform conidia produced in slimy drops from the deep collarettes of pigmented phialides, which occur singly or in clusters. In this study, species in the *Ph. dimorphospora* clade were readily differentiated using ITS and *RPB1* sequences, and several closely related species can be distinguished by morphological characters of asexual states.

Phialocephala scopiformis was the most encountered *Phialocephala* endophyte in our study and the only species found as both an endophyte and saprotroph on tissues of a single host, i.e. needles and fallen decorticated branches of *Picea rubens*. More sampling is required to determine the host and substrate preference of the sexual morph, although it is evident that *Ph. scopiformis* is an endophyte on other conifer host species. White spruce seedlings were successfully colonized by *Ph. scopiformis* after wound inoculation or aerial dispersal of a mycelial suspension, indicating infection may become systemic and not require ascospores (Sumarah et al. 2005). Future work should explore the infection process (e.g. via stomatal invasion) and the growth of infecting hyphae from the needles through the petiole to the vascular system or vice versa. *Phialocephala scopiformis* could provide a good model for such a study, because it was first described from the periderm of living *Picea abies* branches, is routinely isolated as an endophyte of *Picea* needles, and its sexual state was found many times exclusively on fallen *Picea* branches, usually decorticated and exhibiting extensive decomposition.

The ability of many *Phialocephala* species to grow and sporulate at low temperatures indicates that infection of foliage in the early stages of leaf emergence following bud break should be investigated. We selected two *Ph. dimorphospora* strains (DAOMC 87232, DAOMC 250111) to test germination of conidia, because other authors suggested that conidia of some *Phialocephala* spp. are spermatia (Day et al. 2012). Germination of conidia from both strains was observed after 48 h at 15 and 20 °C on both MEA and CMA, indicating that they can function effectively as propagules and are

not strictly spermatia, although a spermatial role cannot be ruled out. The production of conidia in slimy droplets suggests dispersal by insects or rain splash.

Members of the PAC were not isolated from needles nor were their corresponding sexual morphs observed in our study, supporting previous work demonstrating the below-ground niche of this group. Our connections between other *Phialocephala* species and mollisioid apothecia suggests that the collection, description, and sequencing of mollisioid discomycetes in appropriate environments should lead to the discovery of PAC sexual states, the existence of which are suggested by the observations of abortive apothecia by Currah et al. (1993) and mating type loci work by Zaffarano et al. (2010).

The connection between *Phialocephala* and *Mollisia* or mollisioid sexual states was expected because of evidence from phylogenetic studies (Gminder pers. comm., Vrålstad et al. 2002, Grünig et al. 2009, Douglas 2013) and morphological observations by previous authors. Le Gal and Mangenot (1956, 1960, 1961, 1966) made detailed observations of both apothecia and cultural characters of *Mollisia* species and described several conidial states that are consistent with the present generic concept of *Phialocephala* s.l. For example, they described a phialidic morph with deep collarettes ("a transitional form between *Phialophora* and *Cystodendron*") that took at least one year to develop in cultures of *M. coerulans* (Le Gal and Mangenot 1966) and another delayed cystodendron-like state in *M. palustris* cultures. Bubák (1914) described *Cystodendron*, characterized by conidia aggregated in slimy masses from phialides with

deep collarettes and morphologically differentiated from *Phialocephala* by the aggregation of conidiophores into sporodochia. Older (6–8 mo) cultures of *M. discolor* var. *longispora* formed sporodochia composed of densely aggregated phialides with deep collarettes and considered to be cystodendron-like (Le Gal and Mangenot 1956, 1958). Aebi (1972) also described *Cystodendron* states in pure cultures of *Belonopsis ericae*, *Tapesia* (= *Mollisia*) *cinerella*, *T. fusca*, *T. hydrophila*, and *T. villosa*, and considered *Phialocephala* to be morphologically identical to (and therefore synonymous with) *Cystodendron*.

Phialocephala s.l. is still currently polyphyletic, both by including distantly related species and by occurrence in numerous closely related clades intermixed with species named in other genera, some of which have nomenclatural priority (e.g. *Cystodendron*, *Mollisia*, *Trimmatostroma*). Based on ITS sequences, Grünig et al. (2002) showed that *Ph. fusca*, *Ph. humicola* (= *Ph. xalapensis*), and *Ph. virens* are outside the main *Phialocephala* clade. ITS BLAST searches suggest the placement of *Ph. fusca* and *Ph. humicola* in the *Chaetosphaeriaceae*. Morphological and phylogenetic evidence also reveal that *Ph. trigonospora* is not congeneric with *Ph. dimorphospora*, and most likely belongs to *Verticicladiella* (Grünig et al. 2009). *Acephala*, a genus with two described species belonging to the PAC, is congeneric with *Phialocephala* and differentiated only by the lack of observed sporulation in culture (Grünig and Sieber 2005), a taxonomic choice enabled by some interpretations of dual nomenclature but now unacceptable after recent changes to the Code. Although the core clade of *Phialocephala* s.s. is clearly defined as the *Phialocephala dimorphospora* clade, the presence of *Mollisia cinerea*-like

teleomorphs in this clade cast at least some doubt on its nomenclatural priority over *Mollisia* until this species, and the genus, are epitypified (an ongoing project; A. Gminder, pers. comm.). The apparent phylogenetic affinity of the PAC and allied taxa to *Vibrissea* in most phylogenies is also a major taxonomic issue that requires resolution. It is further evident that *Phialocephala* s.s. remains to be comprehensively circumscribed, awaiting reassignment of phylogenetically closely related and unrelated *Phialocephala* species, and the formal transfer of species currently congeneric with *Phialocephala* s.s. (e.g. in *Acephala* and *Mollisia*) to stable and monophyletic genus concepts.

If *Ph. dimorphospora* is indeed congeneric with *M. cinerea*, then nomenclatural decisions will have to consider the interests of users of these names and the independent taxonomic histories of these genera. A principle of the Code is priority of publication. *Mollisia*, described by Karsten in 1871, clearly has priority over *Phialocephala* (1961). Unfortunately, *Mollisia* is not yet robustly circumscribed and the prevailing concept is broad, polyphyletic, and paraphyletic (Crous et al. 2003, Grünig et al. 2009). A definitive nomenclatural conclusion requires at least a preliminary phylogenetic investigation of *Mollisia*. Based on Index Fungorum, 603 names have been applied to *Mollisia*. The number of accepted *Mollisia* species after excluding synonymized taxa and subspecific identifiers is 231, excluding species of other genera that are probably congeneric or that already appear to overlap phylogenetically, such as *Belonopsis*, *Haglundia*, *Nimbomollisia*, *Pyrenopeziza*, and *Tapesia*. Only seven *Mollisia* species are represented in GenBank, including only one representing an ex-type strain, *M. dextrinospora* (better placed in *Pyrenopeziza*, B. Douglas, unpubl.).

Sequencing directly from type specimens, whenever possible, is the preferred course of action. The uncertain location of many types (e.g. the type specimen for *M. cinerea* is lost; A. Gminder, pers. comm.), or their condition or age, significantly tempers optimism for this approach. Species descriptions are often vague by contemporary standards and based on exsiccatae lacking vital characters important to some discomycete taxonomists (Baral 1992). The delineation of *Mollisia*, along with its relationship to *Phialocephala* and the subsequent nomenclatural decisions, are the subject of a concurrent study that should ameliorate some of these important issues.

The issue of unidentified endophytes resulting from culture-based or amplicon-based metagenomic studies can be at least partly resolved by employing an approach using morphology, anatomy, and molecular phylogenetic methods combined with studies of the habitats of these fungi. Endophytes of woody plants are frequently horizontally-transmitted and may not be constrained to colonized host tissue, therefore looking beyond the host or senescent colonized tissue for reproductive morphs should prove fruitful. For example, the discovery of *Dwayaangam colodena*, an aquatic hyphomycete and foliar endophyte of *Picea mariana* and *P. rubens*, provides evidence of a complex aquatic-terrestrial lifecycle that would have remained undiscovered if endophyte researchers restricted their sampling to living host tissue (Sokolski et al. 2006a, Sumarah et al. 2010). Cultural studies of endophytes frequently yield sterile isolates that may be unidentifiable using morphological characters or DNA barcodes, or conversely can produce novel teleomorphs. For instance, Knapp et al. (2015) isolated three novel pleosporalean DSE genera from grass roots and attempted to induce in vitro

sporulation using various methods, including culturing on different media or sterilized plant material, mechanically damaging mycelia, exposure to near-UV light, and slowly drying out cultures. Isolates of *Darksidea* produced ascomata on the surface of stinging nettle stems on synthetic nutrient-poor agar 3–4 wk after inoculation at room temperature.

We recommend a pragmatic strategy to enable confident species identification of taxa combining field mycology, culture isolations, and molecular phylogenetic methods, to bridge the gap between the present dominance of unnamed DNA sequences and the historical literature. This requires epitypification of well-described and distinct species, but also the more laborious but accurate and complete morphological description of novel or imprecisely characterized species, all associated with accessioned DNA sequence data. A concerted effort to sequence types and vouchered specimens of species unrepresented in sequence databases will connect unidentifiable sequences of endophytes with named or describable morphotaxonomic concepts, significantly increase the utility of environmental sequencing and metagenomics tools, and facilitate the development and communication of taxonomic and ecological knowledge (Brock et al. 2009, Nagy et al. 2011).

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AUTHOR CONTRIBUTIONS

JBT conceived and designed the work. JBT, BD, and KAS acquired the data: JBT and KAS provided strains, collections, and sequences from Canadian material, BD provided sequences, observations, images from UK material for comparison; JBT provided the species descriptions, tested all markers and generated all sequences, conducted cultural studies and phylogenetic analyses, created all photographic plates, deposited all data in GenBank and TreeBase, and deposited all cultures in DAOMC and specimens in DAOM. JBT, BD, and KAS analyzed and interpreted the data and taxonomic considerations. JBT and BD drafted the article; JBT, BD, and KAS critically revised the manuscript for content.

Chapter 2: Mollisiaceae: An overlooked lineage of diverse endophytes

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ABSTRACT

Mollisia is a neglected discomycete genus which is commonly encountered as a saprotroph on decaying plant tissues throughout temperate regions. The combination of indistinct morphological characters, more than 700 names in the literature, and lack of reference DNA sequences presents a challenge when working with *Mollisia*. Unidentified endophytes, including strains that produced bioactive secondary metabolites, were isolated from conifer needles in New Brunswick and placed with uncertainty in *Phialocephala* and *Mollisia*, necessitating a more comprehensive treatment of these genera. In this chapter, morphology and multilocus phylogenetic analyses were used to explore the taxonomy of Mollisiaceae, including *Mollisia*, *Phialocephala*, and related genera, using new field collections, herbarium specimens, and accessioned cultures and sequences. The phylogeny of Mollisiaceae was reconstructed and compared using the ITS barcode and partial LSU gene, *RPB1*, *TOP1*, and *LNS2*. The results indicated that endophytism is common throughout the Mollisiaceae lineage in a diverse range of hosts but is rarely attributed to *Mollisia* because of a paucity of reference sequences. Generic boundaries within Mollisiaceae were poorly resolved and based on phylogenetic evidence most genera are polyphyletic. Taxonomic novelties included the description of five novel *Mollisia* species and four novel *Phialocephala* species and the synonymy of *Acephala* with *Phialocephala* and Loramycetaceae with Mollisiaceae.

KEYWORDS

discomycete; molecular taxonomy; *Mollisia*; *Phialocephala*; species identification

INTRODUCTION

In the aptly titled paper, “*Mollisia* in Macaronesia: an exercise in frustration”, Greenleaf and Korf (1980) pointed out, “how little, not how much, we know about *Mollisia* today”, a sentiment that still largely holds true. Taxonomic research on *Mollisia* (Dermateaceae or Mollisiaceae, Helotiales) and related mollisioid discomycetes is notoriously difficult (Greenleaf and Korf 1980, Nauta 2010) and has largely stagnated (but see Tanney et al. 2016a). *Mollisia* is a large, unwieldy genus comprising species that are common saprotrophs in the Northern Hemisphere, usually forming greyish to bluish discoid apothecia on decaying plant tissues.

Apothecia are typically 1–3 mm in diameter, sessile, and characterized by an outer layer (ectal excipulum) composed of pigmented, rounded cells, a hyaline textura intricata inner layer (medullary excipulum), cylindrical paraphyses containing refractive vacuolar bodies, and ascospores that are often 0(–1)-septate, narrowly fusiform, hyaline, and borne in 8-spored euamyloid asci. Axenic cultures may be readily isolated from fresh field collections by allowing ascospores to discharge on standard media such as 2% malt extract agar or potato dextrose agar, although discomycete taxonomists historically did not emphasize cultural studies. The development of mature apothecia in vitro is rarely documented (Gremmen 1955, Tanney et al. 2016a).

Asexual morphs, notably phialocephala-like conidiophores, may develop in vitro (Le Gal and Mangenot 1956) and conidia adapted for aquatic dispersal have also been observed (Webster 1961, Webster et al. 1993). The recent description of diverse

synanamorphs associated with *Phialocephala* has expanded our understanding of the morphological diversity in this lineage (Crous et al. 2015c, Tanney et al. 2016a). Cultural and phylogenetic studies attribute various morphologically-diverse asexual states to *Mollisia* and related genera, including *Anavirga dendromorpha* (Descals and Sutton 1976, Hamad and Webster 1988), *Anguillospora crassa* (Webster 1961), *Casaresia sphagnicola* (Webster and Descals 1975, Webster et al. 1993), *Cheirospora botryospora* (Crous et al. 2015c), *Helicodendron giganteum* (Fisher and Webster 1983), *Paradidymobotryum oblongum* (= *Phialocephala oblonga*) (Tanney et al. 2016a), and *Variocladium giganteum* (Baschien et al. 2013), as well as morphs referable to *Anguillospora*, *Diplococcium*, *Filosporella*, *Septonema*, *Trimmatostroma*, and microsclerotial or sclerotial structures (Webster and Descals 1979, Digby and Goos 1987, Shenoy et al. 2010, Tanney et al. 2016a).

The current understanding of asexual and sexual morphological characters does not permit rapid identification of most *Mollisia* species in the field or even confident identification following detailed microscopic study. Despite difficulties identifying *Mollisia* based on morphology, or perhaps because of such issues, hundreds of species have been named since the description of the type species, *M. cinerea*, in 1871. More than 700 *Mollisia* names exist in the literature and the taxonomic status of many of these species is largely unknown. These numbers do not include possibly congeneric species currently placed in other Dermateaceae genera, such as *Belonidium*, *Belonopsis*, *Haglundia*, *Hysteronaevia*, *Nimbomollisia*, *Niptera*, and *Tapesia*, which were distinguished from *Mollisia* based on morphological characters such as the presence of

long, cylindrical septate marginal hairs (*Haglundia*), a well-developed melanized subiculum (*Tapesia*), septate ascospores with calcium oxalate crystals embedded in the medullary excipulum (*Belonopsis*), or septate ascospores with gelatinous sheathes (*Niptera*) (Nauta and Spooner 2000a, 2000b).

The entangled relationship between *Mollisia* and *Phialocephala* (Vibrissaceae, Helotiales), an asexual morph genus comprising important root and leaf endophytes of a wide variety of hosts, suggests that the ecological function of *Mollisia* species may be more complex than previously assumed (Day et al. 2012, Tanney et al. 2016a). *Phialocephala* species may be plant mutualists, potential biocontrol agents, producers of industrially-significant secondary metabolites, or causal agents of important plant diseases (Frasz et al. 2014, Arneaud and Porter 2015, Wong et al. 2015). Some studies have also reported *Mollisia* as endophytes of leaves and twigs in diverse host plants (Sieber 1989, Barklund and Kowalski 1996, Shamoun and Sieber 2000, Kowalski and Andruch 2012). Close phylogenetic relationships with genera comprising species that inhabit aquatic habitats, including *Loramyces* and *Vibrissea*, provide examples of unexpected ecological and morphological diversity within the lineage (Wang et al. 2006b).

Descriptions of novel *Mollisia* species have largely dwindled in the last 100 years (Fig. 2.1) and while a search for the keyword, "*Mollisia*" in Google Scholar shows a rise in publications containing this term over the last 20 years (Fig. 2.2), most references to *Mollisia* in such publications are cursory, such as biodiversity checklists or accessioned

GenBank sequences. The major obstacles hindering the progress of taxonomic and phylogenetic studies of *Mollisia* include: (1) an absence of authenticated reference sequences; (2) a dearth of ex-type cultures; (3) issues identifying or sequencing exsiccati due to poor condition or loss; (4) difficulty identifying field and herbarium specimens based on indistinct morphological characters; and (5) the absence of a useable taxonomic treatment with identification keys. These obstacles have effectively deterred any concerted effort to confront *Mollisia*, and fewer classical taxonomists and a growing dependence on working with previously identified material or sequences compounds the problem.

Mollisia is an ideal candidate for DNA barcode-based identification and phylogenetic study because it is commonly encountered in temperate regions, easily cultured, and poorly studied. However, as of this writing there are only 80 nuc internal transcribed spacer rDNA (ITS) barcode sequences for purported *Mollisia* species in GenBank. These sequences contain only eight named species (*M. cinerea*, *M. dextrinospora*, *M. fusca*, *M. heterosperma*, *M. incrustata*, *M. melaleuca*, *M. minutella*, and *M. ventosa*) and only one ex-type sequence (*M. dextrinospora*). Of these, sequences of *M. dextrinospora* (NR119489, AY259134, HM116746, JN104537) and *M. incrustata* (GU727556) are not congeneric with *Mollisia* (Bogale et al. 2010, Crous et al. 2014a). Presently, the identification of most described *Mollisia* sp. and related taxa, excluding morphologically-distinct taxa such as *Loramyces* spp. and the well-characterized *Phialocephala* spp., is virtually impossible using sequences.

In this study, the phylogeny of Mollisiaceae is explored using multiple loci and material derived from new field collections, culture collections, and herbaria. The objectives of this study are to: (1) assist users by providing reference data and more comprehensive phylogenies; (2) test *LNS2*, *RPB1*, and *TOP1* as phylogenetic markers and secondary barcodes; (3) provide rationale for the various approaches to fixing the taxonomy of Mollisiaceae; (4) examine the occurrence of endophytism throughout the lineage; and (5) generate interest for this important but neglected group.

MATERIALS AND METHODS

Sampling and isolation of fungi

Field collections of mostly lignicolous apothecia were made in New Brunswick, Ontario, and Quebec, Canada. Herbarium specimens and cultures were kept in the personal collection of the J.B. Tanney and materials of interest were accessioned into the Canadian National Mycological Herbarium (DAOM) and Canadian Collection of Fungal Cultures (CCFC). Other cultures of *Mollisia* and related genera were obtained from the CCFC and the Centraalbureau voor Schimmelcultures (CBS-KNAW) culture collection.

Cultures derived from ascospores were made by suspending mature ascomata from the lid of a 6 cm Petri dish using petroleum jelly or drops of water for up to 12 h and allowing ascospores to eject downward or upward onto the agar surface.

Ascospore isolations were made on 2% malt extract agar (MEA; 20 g Bacto malt extract, Difco Laboratories, Sparks, Maryland; 15 g agar, EMD Chemicals Inc., New Jersey; 1 L

distilled water) or corn meal agar (CMA; Acumedia Manufacturers Inc., Lansing, MI). Monospore cultures were obtained from individual ascospores whenever feasible and transferred to 6 cm Petri dishes containing MEA. Endophyte cultures were isolated following the methods of Tanney et al. (2016a). All cultures were incubated in the dark at 16 °C.

Morphological studies

Vertical sections of apothecia were cut by hand and mounted in either water, 85% lactic acid, Melzer's reagent, 5% KOH, or Lugol's solution with or without 5% KOH pretreatment to test amyloid reactions (Baral 1987). Morphological observations of specimens were made on living material whenever possible. Colony colors were described using the alphanumeric codes of Kornerup and Wanscher (1978). Microscopic measurements were taken from material mounted in water and are presented as ranges calculated from the mean \pm standard deviation of each measured value, with outliers in brackets. Observations were made using an Olympus BX50 light microscope and micrographs were captured using an Evolution MP Color Camera (Media Cybernetics, Silver Spring, CA, U.S.A.) and Image-Pro Plus v6.0 (Media Cybernetics) or an InfinityX-32 camera (Lumenera Corp., Ottawa, Canada) and Infinity Analyze v6.5.2 (Lumenera Corp.) software. Colony macrophotographs were captured with a Nikon Coolpix P5000 (Nikon Inc., Tokyo, Japan) and photographic plates were assembled using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA, U.S.A.).

Attempts to induce sporulation in sterile cultures were made in accordance with Tanney et al. (2016a). In addition, for selected cultures, 0.5–1 mm agar blocks containing mycelia were floated in 9 cm Petri dishes containing ca. of sterile distilled water and incubated for up to 9 mo.

Cardinal temperatures were determined by assessing diameter growth of cultures made by single-point inoculations on MEA and incubated at 5 °C intervals from 5 to 40 °C. Temperature treatments were conducted in triplicate for each isolate and colony measurements were made weekly for 1 mo.

Phylogenetic studies

Total genomic DNA was extracted from 4–12-wk old cultures using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. DNA extractions from herbarium specimens were made using the NucleoMag Trace kit (Macherey-Nagel, Düren, Germany) with an initial tissue grinding stage in liquid nitrogen using an Axygen polypropylene pestle (PES-15-B-SI, Union City, CA, U.S.A.).

The primer pairs ITS1 and ITS4 (White et al. 1990) or ITS4A and ITS5 (Larena et al. 1999) were used to amplify and sequence the ITS region. The *largest subunit of RNA polymerase II (RPB1)* was amplified and sequenced using RPB1-Af and RPB1-6Rlasc (Stiller and Hall 1997, Hofstetter et al. 2007). Partial 18s nuc rDNA (SSU) and 28S nuc rDNA (LSU) genes were amplified and sequenced following the methods of Tanney et al. (2015). Lipin/Ned1/Smp2 (*LNS2*) was amplified using the primers LNS2_468-F and

LNS2_468-R and DNA topoisomerase I (*TOP1*) was amplified using the primers TOP1_501-F and TOP1_501-R (Stielow et al. 2015).

DNA was amplified using a PCR master mix consisting of 0.5 μ L 2 μ M dNTPs, 0.04 μ L 20 μ M forward primer, 0.04 μ L 20 μ M reverse primer, 1 μ L 10 \times Titanium *Taq* buffer (Clontech, Mountain View, CA, U.S.A.), 0.1 μ L 50 \times Titanium *Taq* enzyme (Clontech), 1 μ L of DNA template, and 7.32 μ L sterile Milli-Q water (Millipore, Bedford, MA, U.S.A.) per reaction. For herbarium specimens, 0.5 μ L of 20mg/mL bovine serum albumin (BSA; Thermo Fisher Scientific, Waltham, MA, U.S.A.) was added to each reaction. All loci were amplified using the following PCR profile: 95 $^{\circ}$ C for 3 min, then 35 cycles at 95 $^{\circ}$ C for 1 min, 56 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 1.5 min, followed by a final extension at 72 $^{\circ}$ C for 10 min. *TOP1* and *LNS2* were initially amplified using the Touchdown PCR (68–58 $^{\circ}$ C) as described in Stielow et al. (2015). PCR troubleshooting included adjusting annealing temperature, dilution of DNA, addition of BSA, and using different *Taq* polymerase (Ex *Taq* HS DNA Polymerase, Takara Bio Inc., Otsu, Japan). PCR products were verified by agarose gel electrophoresis and sequenced with Big Dye Terminator (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence contigs were assembled and trimmed using Geneious R8 v8.1.5 (Biomatters, Auckland, New Zealand). Individual gene sequences were aligned using MAFFT v7 and the resulting alignments trimmed and manually checked using BioEdit v7.2.5 (Hall 1999).

To assess individual gene genealogies and apply the genealogical concordance phylogenetic species recognition concept (GCPSR; Taylor et al. 2000) phylogenetic analyses of single gene data sets were performed with Bayesian inference. Alignments consisting of 91 taxa represented by all genes were used to compare gene phylogenies. The partial 18S nuc rDNA region does not provide adequate species resolution for the strains in this study; therefore, the sequences are included as reference data but the phylogeny not shown.

RPB1 and ITS sequences were available for additional species or strains that were excluded from the gene comparison dataset because of failure to amplify one or more gene or were available from GenBank accessions. Additional *RPB1* phylogenies containing 109 taxa and 111 taxa (including *Strossmayeria basitricha* NB-644 and *Variocladium giganteum* CBS 508.71) were analyzed. An ITS phylogeny including 168 taxa was also generated.

The most suitable sequence evolution model for each gene (GTR+I+G) and the ITS GenBank phylogeny (SYM+I+G) was determined based on the optimal Akaike information criterion scores in MrModeltest v2.2.6 (Nylander 2004). Bayesian analysis was performed with MrBayes v3.2 (Ronquist et al. 2012). For each locus, three independent Markov Chain Monte Carlo (MCMC) samplings were performed with four chains (three heated and one cold) with sampling every 500 generations until the standard deviation of split frequencies reached a value <0.01. The first 25% of trees were discarded as burn-in and the remaining trees kept and combined into one

consensus tree with 50% majority rule consensus. Convergence was assessed from the three independent runs using AWTY and Tracer v1.6 (Wilgenbusch et al. 2004, Rambaut et al. 2014). Consensus trees were visualized in FigTree v1.4.2 (Rambaut 2014) and exported as SVG vector graphics for assembly in Adobe Illustrator v10 (Adobe System, San Jose, CA, U.S.A.).

The biodiversity and host, substrate, and biogeographical data of unidentified or environmental ITS sequences related to Mollisiaceae were investigated by downloading relevant sequences from a GenBank BLAST search using the ITS sequence of *Phialocephala dimorphospora* DAOM 87232 as a seed. Sequences were aligned with MAFFT v7 and phylogenetic analysis was conducted with the maximum likelihood (ML) optimality criterion with RAxML v.7.2.8 with the nucleotide model GTR+G, 100 bootstrap replicates and starting with a complete random tree (Stamatakis 2006).

RESULTS

PCR amplification success for DNA obtained from cultures was 100% for ITS, SSU, LSU, and *RPB1*, 97% for *LNS2*, and 93% for *TOP1*. A 56 °C annealing temperature significantly improved *LNS2* and *TOP1* amplification success. The overall success rate of ITS amplification yielding usable sequences from herbarium specimen DNA was 50% (16/32 specimens), which enabled the sequencing of species of mollisoid genera unrepresented in GenBank, including *Hysteronaevia*, *Niptera*, *Nipterella*, *Obtectodiscus*, and *Trichobelonium* (= *Mollisia*). The addition of BSA significantly improved amplification of DNA from herbarium specimens, although this also enhanced

amplification of contaminating fungal DNA in some cases (e.g.: *Candida*, *Cladosporium*, *Malassezia*, *Simplicillium* spp.).

The ITS alignment used in the gene comparison (Fig. 2.3) included 91 taxa and was 652 characters long, containing several insertions resulting in an indel rich alignment around the ITS2 region. The ITS alignment including identified species from GenBank (Fig. 2.4) contained 177 taxa and was 804 characters long. The large ITS alignment with unidentified GenBank sequences included 733 sequences with a length of 616 characters (Fig. 2.5). The initial LSU alignment included 91 taxa and was 1737 characters long with a large ca. 400 bp long insertion region for *M. caesia* (CBS 220.56), *M. discolor* (CBS 289.59), *M. fallens* (CBS 221.56), *M. ventosa* (CBS 322.77), and NB-473. This insertion region was deleted and the final alignment was 1302 bp (Fig. 2.6). Partial *RPB1* fragments were ca. 1200 bp long, with both alignments (Figs. 2.7 and 2.8) ultimately being 822 characters long, containing a small (ca. 20 bp) intron around position 110. *TOP1* fragments were ca. 840 bp long, resulting in an 835 character long alignment with small (<50 bp) introns around positions 200 and 720 (Fig. 2.9). The primer pair used to amplify the hypothetical protein-coding gene *LNS2* yielded short fragments ca. 300 bp long, with a final 281 character long exonic alignment (Fig. 2.10).

To compare phylogenies for each gene, separate analyses were conducted using the 91 taxa represented in all datasets (Figs. 2.3, 2.6, 2.7, 2.9, 2.10). Several incidences of discordance for the relationships of individual taxa were observed among the different gene phylogenies. Notably, *Vibrissea* is basal to Mollisiaceae in the LSU, *RPB1*,

and *TOP1* phylogenies; however, ITS and *LNS2* phylogenies place this genus in the *Phialocephala* s.s. and *Ph. fortinii* sensu lato (s.l.)–*Acephala applanata* complex (PAC) clade. *Phialocephala* s.s. and the PAC are sister in all phylogenies but *TOP1*; excluding this discrepancy, the *TOP1* and *RPB1* phylogenies were generally concordant. The morphologically divergent, semi-aquatic clade comprising *Loramyces*, *Obtectodiscus*, *Ombrophila hemiamyloidea*, and *Mollisia diesbacha* is strongly supported in all phylogenies except *LNS2*. The *LNS2* phylogeny generated more weakly-supported polytomous clades and showed some discordance with the other genes, e.g.: the placement of *P. scopiformis*. Where *RPB1* showed no intraspecific variation, *LNS2* SNPs were observed among strains of *Phialocephala helena* (11 bp difference) and NB334-2C/NB625-7H (4 bp difference). ITS provided a phylogenetic resolution comparable to *RPB1* and *TOP1*, albeit with more weakly supported branches.

The *RPB1* phylogeny (Fig. 2.8) exhibited highly supported branches overall, placing *Vibrissea* outside of the main Mollisiaceae lineage. The backbone support was generally high, although support was moderate [posterior probability (P.P.) = 0.75] between the *Phialocephala* s.s.-PAC and *Mollisia* s.l. clades. *Mollisia dextrinospora* (CBS 401.78) and *M. cinerella* (CBS 312.61) are actually species of *Pyrenopeziza*, a genus that is phylogenetically distinct from the Mollisiaceae lineage. *Mollisia* is polyphyletic, with species spread throughout the lineage shared at least with *Acidomelania*, *Barrenia*, *Cystodendron*, *Hysteronaevia*, *Loramyces*, *Niptera*, *Nipterella*, *Obtectodiscus*, *Ombrophila hemiamyloidea*, *Phialocephala*, and *Trichobelonium*. The terrestrial mollisoid species *Mollisia diesbacha*, described below, is basal in the strongly-supported

semi-aquatic clade comprising *Loramyces* spp., *Obtectodiscus aquaticus*, and *Ombrophila hemiamyloidea*. The recently described asexual root-endophyte genera *Acidomelania* and *Barrenia* are within the lineage; *Barrenia* comprises two polyphyletic species. *Phialocephala* s.s. is sister to the PAC, forming a clade including several endophytic *Phialocephala* and *Mollisia* spp. *Phialocephala scopiformis* is sister to a clade comprising endophytic isolates from *Fagus sylvatica* leaves ("*P. dimorphospora*" CBS 112411) and conifer needles (*Cystodendron dryophilum* CBS 295.81, *Mollisia monilioides*, 625-9A) and apothecial isolates on decaying wood ("*M. cinerea*" CBS 122029, NB-242, NB-648, NB-655). The *RPB1* phylogeny placed *Strossmayeria basitricha* and *Variocladium giganteum* within the lineage based on weakly supported long branches; this placement within the lineage is probably a result of long-branch attraction and resulted in their exclusion from other phylogenetic analyses (Supplementary Figure 2.1).

TAXONOMY

Ombrophila hemiamyloidea produced an unreported phialidic asexual state ca. 1 mo after floating pieces of MEA or OA containing mycelia in water (Fig. 2.11): conidiophores micronematous to macronematous, arising vertically or laterally from mycelium submerged in water, hyaline to subhyaline or light brown, becoming darker with maturity, smooth, cylindrical, thin-walled, 2–3.5 µm diam, up to indeterminate length, with several septa, unbranched or indeterminately branched, forming dense globose conidiogenous heads up to 400 µm diam. Conidiogenous cells phialidic, terminal, sometimes intercalary, cylindrical to ampuliform or subglobose phialides, (9–

)11–14.5(–17.5) × 3–4(–4.5) μm, with deep hyaline to subhyaline cylindrical collarettes, (4–)4.5–5.5(–6) × 2.5–3 μm, occurring singly or in whorls of 2–3(–4) from metulae.

Metulae hyaline to subhyaline or pale brown, cylindrical to doliiform, (4.5–)5–6.5(–7.5) × 3–4(–5) μm. Conidia dimorphic; primary conidia oblong to oblong-ellipsoidal, hyaline, (4.5–)5–6(–6.5) × (1.5–)2 μm; secondary conidia globose to subglobose, hyaline, 2–2.5(–3) × (1.5–)2(–2.5) μm.

Mollisia diesbacha J.B. Tanney, sp. nov. Fig. 2.12

MycoBank MBXXXXXX

Typification. Canada, New Brunswick, Alma, Fundy National Park, East Branch trail, 45.64335 -65.11563, from decaying *Betula alleghaniensis* wood, 25 Sep 2014, J.B. Tanney NB-546 (holotype DAOM XXXXXX). Ex-type culture DAOMC 250732.

Etymology: Named for the characteristic colour (Prussian blue) of the hymenium, in honour of Johann Jacob Diesbach, the chemist who first synthesized Prussian blue.

Colonies 26–28 mm diam after 14 d in the dark at 20 °C on MEA; flat, stellate, with white woolly aerial hyphae toward centre; margin filamentous, diffuse, wide, hyaline; surface white, olive brown in centre (4E6–4F4); reverse white, olive brown in centre (4F6–4F3). Exudates and soluble pigments absent. Mycelium consisting of hyaline to brown, smooth, septate, branched, hyphae 1.5–3 μm diam, sometimes covered with gelatinous sheaths 1–2 μm diam.

Conidiophores micronematous to macronematous, arising vertically or laterally from mycelium submerged in water, pale to dark brown, smooth, cylindrical, thin- or thick-walled, unbranched or with 1–4 series of branches, 2–3.5 μm diam, 20 μm to indeterminate length, with several septa. Conidiogenous cells phialidic, terminal, sometimes intercalary, ampuliform, (7–)9–13.5(–17.5) \times (2.5–)3–4(–4.5) μm , collarettes cylindrical to doliiform, (3–)4–5(–6) \times 2–2.5(–3) μm , hyaline to pale brown, often appearing concolorous with the darker conidiophore, occurring singly or in whorls of 2–5 from metulae. Metulae hyaline to pale brown, cylindrical to obovoid, apices frequently clavate, 6.5–11(–14) \times (2.5–)3–4(–4.5) μm . Conidia dimorphic; primary conidia ellipsoidal to oblong, hyaline, (3–)4–5(–6) \times 2–2.5(–3) μm ; secondary conidia globose, hyaline, 2.5(–3) \times 2–2.5 μm .

Apothecia scattered to gregarious in small groups (2–6); sessile; urceolate to cup-shaped when young, disc plane to concave at maturity; outline entire; dull to dark blue (21D5–21F4), outer surface bluish grey toward base (21F2); 1.5–2.5 mm diam, 0.2–0.4 mm high; margin frequently paler color to white when younger, smooth; subiculum not evident. Ectal excipulum at base and mid flanks textura globulosa to angularis, 60–180 μm thick near base, 20–50 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (14–)15.5–25.5(–34) \times (6.5–)9–13(–14.5) μm ; at upper flank and margin textura angularis to prismatica, composed of globose to obovoid cells with +/- thin walls, (9–)9.5–11.5(–12) \times 7–9 μm ; marginal cells globose to obovoid-clavate, (9–)10–14(–15) \times 5–6.5(–7) μm ; pale to brown (5E4) around margin and becoming greyish brown (6F3) toward base, not gelatinized, crystals or

exudates absent; tissue becoming dark green (27F5) when mounted in 5% KOH. Subicular hyphae sparse, 2.5–3.5 µm diam, thick-walled (0.5–1 µm), hyaline to brownish grey (5E2). Medullary excipulum hyaline, textura intricata, 20–70 µm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 2.5–4 µm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (62–)65–71(–74) × (5–)5.5–7 µm, pars sporifera 25–29 µm, pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (7–)7.5–8(–9) × 2 µm, cylindrical-oblong to cylindrical-fusiform, apices rounded, aseptate, thin-walled, 4–6(–8) guttules (>1–1 µm diam).

Cardinal temperatures: Range 5–35 °C, optimum 20 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Associated with decaying *Betula alleghaniensis* wood.

Distribution: Canada (New Brunswick).

Notes: *Mollisia diesbacha* forms apothecia with a characteristic dull to dark blue hymenium and narrow, cylindrical-oblong to cylindrical-fusiform guttulate ascospores. The phialidic, phialocephala-like asexual state was only observed when agar pieces containing mycelia were floated in sterile water. *Mollisia diesbacha* is phylogenetically basal to a clade containing semi-aquatic species including *Loramycetes juncicola*, *L. macrosporus*, *Obtectodiscus aquaticus*, and *Ombrophila hemiamyloidea*.

Mollisia monilioides J.B. Tanney, sp. nov. Fig. 2.13

Mycobank MBXXXXXX

Typification. Canada, New Brunswick, Northumberland County, Doaktown, 46.480353 -66.058096, isolated as an endophyte from healthy *Picea rubens* needles, 19 Jul 2014, JB Tanney NB-625-6C (**holotype** DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: *monilioides* (Latin), referring to the monilioid conidiophores.

Colonies 15–17 mm diam after 14 d in the dark at 20 °C on MEA; flat with moderately abundant woolly aerial hyphae, fascicular hyphae aggregated in center; margin entire to undulate, diffuse and white; surface dark blond to olive brown (5D4–4D5); reverse brownish grey (5D3). Exudates and soluble pigments absent. Mycelium consisting of hyaline to brown, smooth, septate, branched, hyphae 1.5–3.5 µm diam, sometimes covered with gelatinous sheaths 1–2 µm diam.

Conidiophores micronematous to macronematous, arising vertically or laterally from mycelium submerged in water, pale to dark brown, smooth, cylindrical, thin- or thick-walled, frequently composed of monilioid cells, unbranched or 1–5 series of branches, branching angle usually acute, penicillate or sympodially branched, 2–4 µm diam, up to indeterminate length, with several septa; giving rise to globose or inverted cone-shaped conidiogenous heads or persisting as non-functional conidiophores.

Conidiogenous cells phialidic; terminal, sometimes intercalary; ampuliform, (7.5–)9.5–

12(–13) × (2–)2.5–4 μm; collarettes cylindrical to doliiform or ovoid, 3–4(–4.5) × 2–2.5(–3) μm; hyaline to pale brown, often appearing concolorous with the darker conidiophore; occurring singly, alternately branched from metulae, or in whorls of 2–4 from metulae; phialides sometimes developing percurrently from aperture of proximal phialides or converting into cylindrical non-functional phialides in the sense of Day et al. (2012); Metulae hyaline to pale brown, cylindrical to obovoid, apices frequently clavate with tapered base, (4–) 4.5–7 (–8) × 3–3.5(–4) μm. Conidia dimorphic; primary conidia ellipsoidal to oblong or ovoid, hyaline, 3–4(–5) × 2–2.5(–3) μm; secondary conidia globose to ellipsoidal, hyaline, (2–)2–3(–3.5) × (1.5–)2–2.5 μm; primary conidium is succeeded by secondary conidia, forming false chains that collapse into persistent slimy heads.

Notes: *Mollisia monilioides* is described based on its asexual state and phylogenetic placement within *Mollisia* s.l. Based on sequence data, *M. monilioides* is closely related to other conifer needle endophytes (NB-625-9A from a *Picea rubens* needle and *Cystodendron dryophilum* CBS 295.81 from a *Juniperus communis* needle) and *Mollisia* spp. producing apothecia on decaying wood (*Mollisia cinerea* CBS 122029, NB-655, NB-242). Conidiophores were only observed when MEA blocks with mycelia were floated in sterile water. Structures interpreted as apothecial initials were frequently observed but never formed immature or mature apothecioid structures after 18 mo.

Cardinal temperatures: Range 5–35 °C, optimum 20 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Endophyte of healthy *Picea rubens* needles.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: see Supplementary Table 2.1.

Mollisia novobrunswickia J.B. Tanney, sp. nov.

Fig. 2.14

Mycobank MBXXXXXX

Typification. Canada, New Brunswick, Alma, Fundy National Park, Coppermine trail, 45.5493 -65.01878, from decaying *Betula papyrifera* wood, 27 Sep 2014, J.B.

Tanney NB-580 (holotype DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: Named for the province of New Brunswick, where the fungus was collected.

Colonies 24–26 mm diam after 14 d in the dark at 20 °C on MEA; flat with sparse aerial hyphae; margin diffuse, hyaline; surface white, occasionally sectoring or developing yellowish brown (5E5) concentric rings with age; reverse white. Exudates and soluble pigments absent. Mycelium consisting of hyaline to pale brown, smooth, septate, branched, hyphae 1.5–4 µm diam, sometimes covered with gelatinous sheaths 1–2 µm diam.

Apothecia scattered to crowded and mutually deformed; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plane to concave at maturity; outline entire to undulate or lobate; greyish to dull blue (22B4–23D4), outer surface grey toward base (23E1); 1–2.5 mm diam, 0.2–0.4 mm high; margin frequently paler color, smooth. Ectal excipulum at base and mid flanks *textura globulosa* to *angularis*, 60–175 μm thick near base, 20–48 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (16.5–)17–23(–25.5) \times (7–)11–15.5(–16.5) μm ; at upper flank and margin *textura angularis* to *prismatica*, composed of globose to subglobose or obovoid cells with +/- thin walls, 9–12.5(–16.5) \times (4–)6–8(–9) μm ; marginal cells subglobose to obovoid or clavate, 8–14(–15) \times (4–)5–7.5(–8) μm ; pale to yellowish brown (5D5) around margin and becoming greyish brown (5F3) toward base, not gelatinized, crystals or exudates absent; tissue becoming dark green (27F5) when mounted in 5% KOH. Subicular hyphae sparse, 2.5–3.5 μm diam, thick-walled (0.5–1 μm), dark brown (6F4). Medullary excipulum hyaline, *textura intricata*, 30–60 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, (2.5–)3–4 μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction negative. Asci arising from croziers, cylindrical-clavate, 8-spored, 50–58(–65) \times (5–)6–8(–9) μm , pars sporifera 20–24 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (6–)7–9(–9.5) \times 2–3 μm , ellipsoidal to oblong, apices rounded, aseptate, thin-walled, small (>1) guttules sparsely present.

Notes: Apothecia of *Mollisia novobrunswickia* were commonly encountered on decaying *Betula* wood in New Brunswick and are characterized macroscopically by a greyish to dull blue hymenium. This species is probably closely related to *Mollisia cinerea* s.s. and shares similar (probably insignificant and variable) features described by Batsch (1786), including apothecia that become somewhat pulvinate with age and a sinuate-lobate, scorbiculate and crisped margin (at least when immature). Unlike *M. cinerea*, the hymenium of *M. novobrunswickia* is bluish versus cinereous and does not dry to a dirty white colour. The margin of *M. novobrunswickia* often appears white or pale in colour in younger specimens and some older specimens; although other authors such as Persoon (1799) and Karsten (1871) described a white margin, this feature is absent from Batsch's description. The ascospores dimensions of *M. cinerea* according to Karsten (1871) occupy a large range (5–12 × 1–2.5 µm) and are shorter than those of *M. novobrunswickia*.

Cardinal temperatures: Range 5–35 °C, optimum 20–25 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Associated with decaying *Betula alleghaniensis* and *B. papyrifera* wood and Endophyte of healthy *Abies balsamea* needles.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: 447, 579, 580, 238-2A, 732

Mollisia prismatica J.B. Tanney, sp. nov. Fig. 2.15

Mycobank MBXXXXXX

Typification. Canada, Quebec, Gatineau (Aylmer), Jardin Lavigne Park, 45.418969
-75.834870, from decaying *Acer saccharum* wood, 14 Sep 2015, J.B. Tanney NB-688
(holotype DAOM 696477). Ex-type culture DAOMC XXXXXX.

Etymology: *prismatica* (Latin), referring to the large crystals present in the inner
ectal excipulum and medullary excipulum.

Colonies 7–10 mm diam after 14 d in the dark at 20 °C on MEA; flat to slightly
convex, moderately abundant woolly aerial hyphae; margin entire, reddish grey (7B2);
surface dark brown to dark ruby (7F4–12F3); reverse reddish grey (12F2). Abundant
light yellow (3A5), up to 2 mm diam, flat, dendritic or acicular crystals on colony surface
and surface of surrounding agar, faint pale yellow (3A3) soluble pigment present in
surrounding agar. Mycelium consisting of hyaline to pale brown, smooth, septate,
cylindrical but sometimes sinuous in outline, often constricted at septa, branched,
hyphae 1.5–4 µm diam, containing abundant oily guttules.

Apothecia scattered to gregarious in small groups (2–4); sessile; subiculum not
evident; urceolate to cup-shaped when young, disc plane to pulvinate at maturity;
outline entire; white to pale yellow (4A3), outer surface greyish yellow toward base
(4B3); 0.7–1.75 mm diam, 0.2–0.5 mm high; margin frequently paler color, smooth. Ectal
excipulum at base and mid flanks textura globulosa to angularis, 60–100 µm thick near

base, 25–42 μm thick towards margin; at upper flank and margin textura angularis to prismatica, composed of globose to elongated clavate cells with +/- thin walls, (8–)8.5–10.5(–11.5) \times 6–8 μm ; marginal cells globose to obovoid, (9–)10–15 \times (5–)6–8.5(–11) μm ; pale to greyish yellow (4B3) around margin and becoming brownish orange (5C5) toward base, not gelatinized, abundant crystals in inner ectal and medullary excipula, rhomboidal to amorphous, 3.5–16 μm diam; tissue becoming dark green (27F5) when mounted in 5% KOH. Subicular hyphae sparse, 2.5–3.5(–4) μm diam, thick-walled (0.5–1 μm), hyaline to light brown (5D4). Medullary excipulum hyaline, textura intricata, 80–200 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–4.5 μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (65–)67–80(–82) \times (5–)6–8(–9) μm , pars sporifera 24–45 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, 10–11.5(–12.5) \times 3.5–4(–4.5) μm , ellipsoidal-fusiform to cylindrical-clavate, apices rounded, aseptate, thin-walled, small (>1–1.5 μm diam) guttules sparsely present.

Notes: *Mollisia prismatica* is distinguished by its white to pale yellow hymenium, thick medullary excipulum, abundant crystals in the inner ectal and medullary excipulum, and broad ascospores. Several lignicolous *Mollisia* species with white to pale hymenia have been described: (i) *Mollisia melaleuca* differs from *M. prismatica* by the spore dimensions (8 \times 2 μm) and dark ectal excipulum; (ii) *M. sublividula* differs by the smaller asci (32–42 \times 4.5–5.5 μm) and ascospores (4–7 \times 1.5 μm); (iii) *M. glenospora*

differs by its diminutive apothecia (0.25–0.5 mm), marginal hairs, and larger ascospores (12–15 × 7–8 µm); and (iv) *M. caespiticia* differs by the smaller asci (30–40 × 3–4.5 µm) and ascospores (4–6 × 1–1.5 µm). *Mollisia prismatica* is morphologically referable to species previously or currently placed within *Belonopsis* because of its white to pale yellow hymenium, less pigmented or pale ectal excipulum, presence of crystals in the inner ectal and medullary excipulum, and pulvinate apothecia. However, most *Belonopsis* species are graminicolous with longer ascospores that are frequently multiseptate when young. *Trichobelonium kneiffii* (= *Belonopsis retincola*) is graminicolous (*Phragmites*) with apothecia that contain abundant excipular crystals, long guttulate ascospores 12.5–28 × 2–3 µm, and frequently occur on a subiculum.

Cardinal temperatures: Range 5–30 °C, optimum 25–30 °C, minimum <5 °C, maximum slightly >30 °C.

Host range: Associated with decaying *Acer saccharum* wood.

Distribution: Canada (Quebec).

Additional specimens and cultures examined: see Supplementary Table 2.1.

Mollisia ravum J.B. Tanney, sp. nov. Fig. 2.16

Mycobank MBXXXXXX

Typification. Canada, New Brunswick, Alma, Fundy National Park, Coppermine trail, 45.5493 -65.01878, from decaying *Betula alleghaniensis* wood, 27 Sep 2014, J.B.

Tanney NB-584 (holotype DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: Named for the greyish colour of the hymenium.

Colonies 11–14 mm diam after 14 d in the dark at 20 °C on MEA; flat with sparse aerial hyphae; margin wide, undulate, hyaline; surface greyish orange (5B5) and yellowish brown (5F4), occasionally sectoring; reverse brownish orange to brown (5C4–5E4). Exudates and soluble pigments absent. Mycelium consisting of pale brown to brown, smooth, septate, branched, hyphae 1.5–3.5 µm diam, sometimes covered with thin (> 1 µm) yellow to deep orange (4A8–6A8) crystalline sheath or dark brown (5F8) exudate up to 5 µm diam.

Apothecia scattered to gregarious or caespitose; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plane to concave or at maturity; outline entire to undulate; dull blue to bluish grey (21D4–21F2), outer surface darker base; 1–2 mm diam, 0.2–0.3 mm high; margin smooth, sometimes appearing crenulate. Ectal excipulum at base and mid flanks textura globulosa to angularis, 60–130 µm thick near base, 25–50 µm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (13–)14–18.5(–19.5) × (10–)10.5–12(–13) µm; at upper flank and margin textura angularis to prismatica, composed of globose to subglose or obovoid cells with +/- thin walls, (6–)7–9(–11.5) × (4.5–)5–7(–8) µm; marginal cells subglobose to obovoid or clavate, 8.5–12(–13.5) × 4–6(–7) µm; brownish orange (5C3) around margin and becoming brown (5F5) toward base, not gelatinized, crystals or exudates absent; tissue becoming greenish grey (28F2) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, (2.5–)3–3.5(–4) µm diam, thick-walled

(0.5–1 µm), dark brown (6F4). Medullary excipulum hyaline, textura intricata, 18–40 µm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–4 µm wide, containing large highly refractive vacuole bodies when living; not exceeding mature asci. KOH reaction negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (54–)56–62(–65) × (4.5–)5–6 µm, pars sporifera 25–35 µm, pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (6.5–)7–9(–10) × 2–2.5(–3) µm, oblong, allantoid to slightly sigmoidal, one end sometimes more tapered or curved, apices rounded, aseptate, thin-walled, (1–)2–4(–7) small (<1–1 µm diam) guttules present. All measurements made with rehydrated specimens.

Cardinal temperatures: Range 5–35 °C, optimum 20 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Associated with decaying *Betula alleghaniensis* wood.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: NB-369.

Notes: *Mollisia ravum* is characterized by apothecia with dull blue to bluish grey hymenia. Based on the current phylogenetic analyses, it is closest related to *Mollisia melaleuca* CBS 589.84 and unidentified *Mollisia* endophytes of *Picea rubens* needles (NB-625-7H, NB-334-2C) and is probably related to the *Mollisia cinerea* s.s. clade.

Phialocephala amethystea J.B. Tanney, sp. nov. Fig. 2.17.

Mycobank MBXXXXXX

Typification. Canada, New Brunswick, Alma, Fundy National Park, Maple Grove trail, 45.58178 -64.98633, from fallen *Acer saccharum* branch, 16 Jul 2014, J.B. Tanney NB-469 (holotype DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: Named for the purplish colour of the large crystals produced abundantly on the surface and below the agar in cultures grown on MEA.

Colonies 30–35 mm diam after 14 d in the dark at 20 °C on MEA; flat, slightly convex with sparse woolly aerial hyphae toward centre; margin filamentous, diffuse, wide, hyaline; surface olive brown (5F5); reverse greyish brown (5F3). Exudates and soluble pigments absent. Abundant cherry red to deep magenta (10B8–13D8), up to 400 µm diam, acicular crystals on colony surface, surface of surrounding agar, and submerged in agar.

Mycelium consisting of hyaline to subhyaline or brown, smooth, septate, branched, guttulate, hyphae 2–4 µm diam, thin-walled or thick-walled (up to 1 µm thick), sometimes covered with entire or sinuate gelatinous sheaths 1–3 µm diam or encrusted with cherry red to ruby (10B8–12D12) thin crystalline sheath or acicular crystals.

Conidiophores micronematous to macronematous, occasionally reduced to conidiogenous cells, arising vertically or laterally from mycelium, hyaline to dark brown,

smooth, cylindrical, thin- or thick-walled, older conidiophores sometimes covered in 0.5–2 µm wide gelatinous sheath, unbranched or 1–3 series of branches, often branching from base, (2.5–)3–4 µm diam, 25–46(–55) µm tall, with several septa; giving rise to flabellate to globose conidiogenous heads. Conidiogenous cells phialidic; terminal, sometimes intercalary; ampuliform to ellipsoidal, (7.5–)10–13(–14.5) × (2.5–)3–4(–4.5) µm; collarettes deep, cylindrical to doliiform with apex sometimes flaring, (2.5–)3–4(–7) × (2–)2.5–3(–4) µm; hyaline to pale brown or brown, becoming thick-walled, darker, septate, and swollen with age; occasionally occurring singly, usually in whorls of 3–5(–7) from metulae. Metulae pale to brown, cylindrical to broadly clavate, (3.5–)4–7 (–10) × (2.5–)3–4(–5) µm. Conidia dimorphic; primary conidia bullet-shaped to elongate-pyriform or ossiform, base often truncate, hyaline, (3–)3.5–4.5(–6) × 1.5–2 µm; secondary conidia globose, base often protruding and truncate, hyaline, 2–2.5 × 2–2.5 µm; primary conidium is seceded by secondary conidia, forming false chains that collapse into persisting slimy heads.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Associated with decaying *Acer saccharum* wood and endophyte of healthy *Picea rubens* needles.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: NB-382-4F

Notes: *Phialocephala amethystea* is most closely related to *P. compacta* based on sequence data and shares some morphological similarities including phialides, collarettes, conidia, and conidiogenous heads of comparable dimensions and conidiogenous heads that become sclerotized with age; Kowalski and Kehr (1995) mentioned the occasional formation of crystals in *P. compacta* cultures but did not provide details. *Phialocephala amethystea* and *P. compacta* share 95% similarity for *RPB1* sequences and 94% similarity for ITS sequences.

Phialocephala biguttulata J.B. Tanney, sp. nov. Fig. 2.18

Mycobank MBXXXXXX

Typification. Canada, Ontario, Ottawa, Saddlebrook Estates, South of John Aselford Drive, 45.375583 -76.04995, from decaying stem of large wind-fallen *Pinus strobus*, 17 Jun 2014, K.A. Seifert NB-649 (holotype DAOM XXXXXX). Ex-type culture DAOMC 250754.

Etymology: *biguttulata* (Latin), referring to the two large guttules in the ascospores.

Colonies 23–25 mm diam after 14 d in the dark at 20 °C on MEA; convex with woolly aerial hyphae; margin diffuse, hyaline; surface soot brown to dark brown (5F5–6F3); reverse brownish grey (7F2). Exudates and soluble pigments absent. Mycelium consisting of subhyaline to brown, smooth, septate, branched, hyphae 2–5.5 µm diam,

thin- or 1–1.5(–2) μm thick-walled, aerial mycelia friable, sometimes covered with exudate layer 2–4(–6.5) μm diam.

Apothecia scattered to gregarious; sessile; subiculum not evident; urceolate to cup-shaped when young, disc plane to concave at maturity; outline entire; brownish grey to bluish grey (5D2–20D3), outer surface darker; 0.7–2 mm diam, 0.2–0.3 mm high; margin frequently paler color, smooth. Ectal excipulum at base and mid flanks textura globulosa to angularis, 40–95 μm thick near base, 19–34 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (13–)14–20(–23) \times (7–)9–12.5(–13) μm ; at upper flank and margin textura angularis to prismatica, composed of globose or cylindrical to elongated clavate cells or with +/- thin walls, 13–20(–22.5) \times (5.5–)7–9 μm ; marginal cells cylindrical to obovoid or clavate, (10–)11–17(–20) long, maximum width towards apex 6–8.5(–10) μm , minimum width at base (4–)4.5–6 μm ; brownish orange to brown (5D4–6E4) around margin and becoming dark brown (6F7) toward base, not gelatinized, crystals or exudates absent; tissue becoming dark green (27F5) when mounted in 5% KOH. Subicular hyphae sparse, 2.5–4 μm diam, thick-walled (0.5–1 μm), dark brown (5F8). Medullary excipulum hyaline, textura intricata, 20–34 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–3.5 μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction negative. Asci arising from croziers, cylindrical-clavate, 8-spored, (53–)58–72(–76) \times (6–)6.5–7.5 μm , pars sporifera 18–27 μm , pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely

uniseriate, (7.5–)8–9(–10) × 3–3.5(–4) μm, ellipsoidal-fusiform to oblong, apices rounded, aseptate, thin-walled, frequently guttulate with two polar guttules (1.5–2 μm diam).

Cardinal temperatures: Range 5–35 °C, optimum 25–30 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Associated with decaying *Pinus strobus* wood.

Distribution: Canada (Ontario).

Additional specimens and cultures examined: see Supplementary Table 2.1.

Notes: *Phialocephala biguttulata* is distinguished from other species in the *Phialocephala dimorphospora* s.s. species complex by the two large (1.5–2 μm diam) guttules that occur towards both poles of the ascospores.

Phialocephala collarifera J.B. Tanney, sp. nov. Fig. 2.19

Mycobank MBXXXXXX

Typification. CANADA. QUEBEC: Aylmer, Jardin Lavigne Park, 45.418969 - 75.834870, from decaying *Betula papyrifera* log, 29 Aug 2015, J.B. Tanney/B. Tanney NB-683, (holotype DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: *collarifera* (L.), bearing collars, to describe the deep collarettes.

Colonies 28–30 mm diam after 14 d in the dark at 20 °C on MEA; flat to slightly convex with moderately abundant woolly aerial hyphae; margin diffuse, wide, hyaline;

surface olive brown to greyish brown (4F4–6F3); reverse dark brown to brownish grey (6F4–6F2). Exudates and soluble pigments absent. Mycelium consisting of subhyaline to brown, smooth, septate, branched, hyphae 2.5–5 μm diam, thin- or thick- (1 μm) walled, sometimes covered with gelatinous sheaths 1.5–4.5 μm diam. Conidiophores very abundant among aerial hyphae.

Conidiophores micronematous to macronematous, arising vertically or laterally from mycelium, pale to dark brown, smooth, cylindrical, thin- or thick-walled, older conidiophores sometimes covered in 1–2.5 μm wide gelatinous sheath, unbranched or 1–2 series of branches, branching angle usually acute, (2.5–)3–4 μm diam, (30–)39–88(–125) μm tall, with several septa; giving rise to globose conidiogenous heads. Conidiogenous cells phialidic; terminal, sometimes intercalary; ampuliform, (13.5–)16.5–21.5(–24) \times (2.5–)3–3.5 μm ; collarettes deep, cylindrical with slightly flaring apex, (5–)6.5–8(–9) \times (2–)2.5–3 μm ; hyaline to pale brown, often appearing concolorous with the darker conidiophore; occasionally occurring singly, usually in whorls of 2–4(–5) from metulae. Metulae pale to brown, cylindrical to clavate, (8–)9.5–13.5 (–15) \times (2.5–)3–4 μm . Conidia dimorphic; primary conidia elongate-ellipsoidal to elongate-pyriform, hyaline, (6.5–)7–8.5(–9.5) \times 2–2.5 μm ; secondary conidia ellipsoidal to oblong or obovoid, one end sometimes more tapered or subtruncate, hyaline, (3–)3.5–4.5(–5.5) \times 2–2.5(–3) μm ; primary conidium is seceded by secondary conidia, forming false chains that collapse into persisting slimy heads.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: On decaying *Betula papyrifera* wood.

Distribution: Canada (Quebec).

Additional specimens and cultures examined: see Supplementary Table 2.1.

Notes: *Phialocephala collarifera* is a member of the *Phialocephala dimorphospora* s.s. clade and is closely related to *P. dimorphospora*. It differs morphologically from *P. dimorphospora* by its longer phialides, (13.5–)16.5–21.5(–24) × (2.5–)3–3.5 µm vs. 11–17.5 × 2.5–3 µm (*P. dimorphospora*), longer collarettes, (5–)6.5–8(–9) × (2–)2.5–3 µm vs. 3–5.5 × 2.5–3 µm (*P. dimorphospora*), larger primary conidia, (6.5–)7–8.5(–9.5) × 2–2.5 µm vs. 3.5–5.5 × 2.5–3 µm (*P. dimorphospora*), and larger secondary conidia (3–)3.5–4.5(–5.5) × 2–2.5(–3) µm vs. 2–2.5 × 2–2.5 µm (*P. dimorphospora*).

Phialocephala helena J.B. Tanney, sp. nov. Figs. 2.20, 2.21

Mycobank MBXXXXXX

Typification. Canada, New Brunswick, Alma, Fundy National Park, Maple Grove trail, 45.58178 -64.98633, from decaying *Acer saccharum* branch, 16 Jul 2014, *H.M. Spizarsky & J.B. Tanney NB-467 (holotype DAOM XXXXXX)*. Ex-type culture DAOMC 250756.

Etymology: Named for the collector of the type specimen, H.M. Spizarsky.

Colonies 20–28 mm diam after 14 d in the dark at 20 °C on MEA; flat, sparse aerial hyphae, fascicular hyphae aggregated in center; margin entire, hyaline; surface brown to greyish brown (5E4–6F3); reverse greyish brown to brownish grey (5F3–5F2). Exudates absent, greyish yellow (4B4) soluble pigment sometimes present in surrounding agar. Mycelium consisting of subhyaline to brown, smooth, septate, branched, hyphae 1.5–4 µm diam, thin- or thick- (1 µm) walled, sometimes covered with gelatinous sheaths 1–3 µm diam.

Conidiophores micronematous to macronematous, arising vertically or laterally from mycelium, subhyaline to dark brown, smooth, cylindrical, thin- or thick-walled, older conidiophores sometimes covered in 1–3 µm wide gelatinous sheath, unbranched or 1–3 series of branches, 2.5–3(–3.5) µm diam, 25–110 µm tall, with several septa; giving rise to inverted cone-shaped conidiogenous heads. Conidiogenous cells phialidic; terminal, sometimes intercalary; ampuliform to ellipsoidal with age, (11–)13–18(–21.5) × (2–)2.5–3.5(–4) µm; collarettes deep, cylindrical sometimes with slightly flaring apex, (3–)4–7(–8) × 2–3 µm; hyaline to brown; occasionally occurring singly, usually in whorls of 2–4 from metulae. Metulae hyaline to brown, cylindrical to clavate, (6.5–)7.5–11 (–12) × (2–)2.5–3.5(–4) µm. Conidia dimorphic; primary conidia elongate-ellipsoidal to elongate-pyriform, hyaline, 3.5–4.5(–5.5) × 1.5–2(–2.5) µm; secondary conidia globose, base rounded to protruding and truncate, hyaline, 2(–2.5) × 2(–2.5) µm; primary

conidium is seceded by secondary conidia, forming false chains that collapse into persisting slimy heads.

Apothecia scattered to gregarious in small groups; sessile; subiculum not evident; occasionally erumpent; urceolate to cup-shaped when young, disc plane to concave at maturity; outline entire to undulate or lobate; pale blue to dull blue (21A3–21D4) when young, becoming greyish blue (21E5), hymenium often white in centre or patches; outer surface dark brown toward base (7F4); 1–2 mm diam, 0.25–0.4 mm high; margin frequently paler color, smooth. Ectal excipulum at base and mid flanks textura globulosa to angularis, 50–200 μm thick near base, 20–40 μm thick towards margin, composed of globose to isodiametric cells with thin to slightly thickened walls, (7–)9–13(–15) \times (5.5–)7–10(–11) μm ; at upper flank and margin textura angularis to prismatica, composed of globose to elongated clavate cells with +/- thin walls, (7–)9.5–15(–18) \times (7–)8–11(–13) μm ; marginal cells globose to elongate-obovoid or clavate, (7–)12–20 \times (5–)6–8(–9) μm ; pale to greyish yellow or greyish red (4B5–8D5) around margin and becoming dark brown (7F4) toward base, not gelatinized, crystals or exudates absent; tissue becoming dark green (27F5) when mounted in 5% KOH. Subicular hyphae sparse to moderately abundant, 2.5–3.5 μm diam, sometimes thick-walled (0.5–1 μm), light to dark brown (5D4–7F4). Medullary excipulum hyaline, textura intricata, 27–72 μm thick. Paraphyses cylindrical with rounded apices, septate, simple, thin-walled, 3–4(–4.5) μm wide, containing large highly refractive vacuole bodies; not exceeding mature asci. KOH reaction strong, paraphyses turning yellow (3A6), visible with unaided eye. Asci arising from croziers, cylindrical-clavate, 8-spored, (55–)64–76(–

76.5) × (6–)7–8 (–9) μm, pars sporifera 21–29 μm, pore amyloid in Melzer's reagent or Lugol's solution with 5% KOH pretreatment (euamyloid), protoplasm turning brick red (7D7) in Lugol's solution. Ascospores biseriate to obliquely uniseriate, (9.5–)11–13.5(–15.5) × (2.5)3–3.5(–4) μm, oblong to oblong-fusiform, straight, occasionally curved or clavate on one end, apices rounded, aseptate, thin-walled, >1–1.5 μm diam guttules aggregated at both poles.

Cardinal temperatures: Range 5–35 °C, optimum 25 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Endophyte of healthy *Picea mariana* and *P. rubens* needles and associated with decaying *Acer saccharum* and *Betula alleghaniensis* wood.

Distribution: Canada (Ontario and New Brunswick).

Additional specimens and cultures examined: see Supplementary Table 2.1.

Notes: *Phialocephala helena* differs from the closely related *P. piceae* by its grayish blue hymenium colour, longer ascospores (9.5–)11–13.5(–15.5) μm vs. (7.5–)9–12(–15) μm (*P. piceae*), and larger asci (55–)64–76(–76.5) × (6–)7–8 (–9) μm vs. (33–)37–49(–53) × 4–7 (*P. piceae*). The conidiophores of *P. helena* are longer and more complexly branched than those of *P. piceae* and the phialides of *P. helena* have larger collarettes (3–)4–7(–8) × 2–3 μm than *P. piceae* (3–4 × 2–2.5 μm). Both *P. helena* and *P. piceae* produce apothecia exhibiting a strong yellow KOH reaction, are occasionally

erumpent, and are *Picea* needle endophytes also found in association with decaying hardwood.

Phialocephala vermiculata J.B. Tanney, sp. nov. Fig. 2.22

MycoBank MBXXXXXX

Typification. Canada, New Brunswick, Sunbury County, Acadia Research Forest, 45.996125 -66.303769, isolated as an endophyte from a healthy *Picea glauca* needle, Jun 1985, J.A. Findlay and J.D. Miller 4GP4C2 (holotype DAOM XXXXXX). Ex-type culture CBS 120378; DAOMC 229535.

Etymology: Named for the production of the macrocyclic dilaetone vermiculin by the type strain.

Colonies 30–32 mm diam after 14 d in the dark at 20 °C on MEA; flat to slightly convex with moderately abundant aerial hyphae; margin entire, wide, hyaline; surface yellowish brown to sepia (5E5–5F4); reverse yellowish brown to brownish grey (5E5–6F2). White plumose crystals up to 12 mm long forming on surface or submerged below agar surface often at colony margin, composed of acicular crystals up to 5 µm diam. Exudates and soluble pigments absent. Mycelium consisting of subhyaline to brown, smooth, septate, branched, hyphae 2–4 µm diam, sometimes covered with exudates 1.5–4.5 µm diam.

Host range: Endophyte of healthy *Picea glauca* needle.

Distribution: Canada (New Brunswick).

Notes: *Phialocephala vermiculata* is phylogenetically closest related to a strain identified as *Mollisia ligni* var. *olivascens* CBS 291.59 and is sister to the *Phialocephala dimorphospora* s.s. clade. This species is based on a single strain, DAOMC 229535, which forms large plumose crystals and has so far not been induced to sporulate despite long-term incubation (up to 24 mo) at 5–30 °C on CMA, MEA, OA, WA with or without the addition of sterile filter paper, and floating agar blocks containing mycelia in sterile water for up to 10 mo. *Phialocephala vermiculata* DAOMC 229535 produces the antiinsectan and antifungal macrocyclic dilaetone vermiculin in vitro and in inoculated *Picea glauca* needles.

New combination for *Mollisiaceae*

Mollisiaceae Rehm [as 'Mollisieae'], in Winter, Rabenh. Krypt.-Fl., Edn 2 (Leipzig) 1.3(lief. 35): 503 (1891) [1896]

Mycobank MB81017; Index Fungorum IF81017

= *Loramycetaceae* Dennis ex Digby & Goos, Mycologia 79(6): 829 (1988) [1987]

New combinations for *Phialocephala*

Phialocephala applanata (Grünig & T.N. Sieber) J.B. Tanney, comb. nov. MycoBank MBXXXXXX.

Basionym: *Acephala applanata* Grünig & T.N. Sieber, Mycologia 97(3): 634 (2005)

Phialocephala macrosclerotiora (Münzenb. & Bubner) J.B. Tanney, comb. nov.

Mycobank MBXXXXXX.

Basionym: *Acephala macrosclerotiorum* Münzenb. & Bubner, Mycorrhiza 19: 487 (2009)

New combinations for *Pyrenopeziza*

Pyrenopeziza dextrinospora (Greenleaf & Korf) J.B. Tanney, **comb. nov.** Mycobank MB
MBXXXXXX.

Basionym: *Mollisia dextrinospora* Korf, in Greenleaf & Korf, Mycotaxon 10(2): 462 (1980)

DISCUSSION

Phylogenetic markers and barcodes

Most of the scant *Mollisia* sequences currently in GenBank are of rDNA genes, with few protein-coding gene sequences available. In this study, three standard rDNA genes (ITS, SSU, and LSU) and three protein-coding genes (*LNS2*, *RPB1*, *TOP1*) were sequenced to explore phylogenies based on unlinked gene genealogies, test species concepts through the application of the GCPSR concept, assess potential secondary barcode markers, and generate reference sequences. The ITS barcode is readily amplified in all taxa included in this study and sufficiently resolves most species, although variation between members of the PAC and species such as *P. catenospora* and *P. nodosa* is low (e.g.: 4 bp difference between *P. catenospora* and *P. nodosa*).

Preliminary attempts to sequence various protein-coding genes from Mollisiaceae using standard primers, included actin (Carbone and Kohn 1999), beta-tubulin (Glass and Donaldson 1995, O'Donnell and Cigelnik 1997), phosphoglycerate kinase (Stielow et al. 2015), *Mcm7* and *Tsr1* (Schmitt et al. 2009), and *RPB2*, were very discouraging (data not shown). Using the high fidelity primer pair RPB1-Af and RPB1-

6Rlasc, *RPB1* amplifies readily and provides unambiguous exon alignments and good species resolution and phylogenetic signal. Stielow et al. (2015) identified *LNS2*, *PGK*, and *TOP1* as promising supplementary barcodes with higher resolution than ITS. For example, *PGK* and *TOP1* resolved related *Fusarium* and *Penicillium* species as well as partial β -tubulin II (*TUB2*) and translation elongation factor 1- α (*TEF1 α*). *PGK* was abandoned early in this study after amplification failure during the preliminary screening of potential secondary barcodes, although future work may include the testing of other primer sets (e.g.: PGK533; Stielow et al. 2015). *TOP1* sufficiently delineated species and the resulting phylogeny showed comparable topology and branch posterior probability support values as the *RPB1* phylogeny. The phylogeny resulting from the *LNS2* alignment was more weakly supported than the other gene phylogenies, with several clades forming polytomic groupings. Significant discrepancies between *LNS2* and other gene phylogenies include the placement of *P. scopiformis* sister to *Vibrissea* and *Tapesia rosae* placed within the *P. dimorphospora* s.s. and PAC clade. Species resolution was overall satisfactory for *LNS2*, although pronounced intraspecific variation was observed in some species.

Sequencing Mollisiaceae specimens or isolates should at least include ITS, *RPB1*, and LSU for identification and phylogenetic reconstruction. Additional supplementary barcodes that were untested in the present study but show promise in other fungal groups, such as *TEF1 α* , should also be considered. There are a significant number of reference sequences available for the readily amplifiable rDNA genes and species resolution and phylogenetic signal from ITS is satisfactory. *RPB1* was readily amplified,

provided a strongly supported phylogeny with good species resolution, and there are a growing number of reference sequences available for this gene for other fungi. While LSU is more conserved than ITS and *RPB1*, providing lower resolution at the species rank, LSU sequences are represented by abundant reference sequences, provide good generic or higher level taxonomic classification, and may be aligned across distantly related taxa, which is useful for estimating phylogenies of communities and placing new fungal lineages or analyzing basal lineages (Liu et al. 2012, Porter and Golding 2012). While *TOP1* performed well in terms of amplification, phylogenetic signal, and interspecific sequence divergence, there are few reference sequences available for this gene in GenBank. Stielow et al. (2015) reported that *LNS2* was a promising secondary barcode for basidiomycetes such as the Pucciniomycotina, but performance of *LNS2* among the Ascomycota was insufficiently tested and therefore could not be thoroughly assessed. While *LNS2* was readily amplified and generally provided good interspecific variation, the data in this study do not provide strong support for the use of *LNS2* in phylogenetic analyses of this lineage. The short fragment length of *LNS2* sequences may be desirable for barcoding herbarium specimens that contain degraded or fragmented DNA or for barcoding environmental samples; however, the poorly resolved polytomies and high intraspecific variation observed in this study warrant caution and more inquiry for its use in phylogenetic reconstruction. However it should be noted that *LNS2* and *TOP1* were proposed by Stielow et al. (2015) as barcodes and not phylogenetic markers. Species identification using *LNS2* and *TOP1* is currently infeasible because of a lack of available reference sequences.

Endophytism throughout Mollisiaceae

Mollisiaceae is a large, diverse lineage containing an exceptional number of apparently facultative endophytes isolated from the roots, foliage, and cambium of various plant hosts worldwide. Current sampling is too scant to allow recognition of overall host preference or biogeographical patterns among endophyte taxa; however, the co-occurrence of the same or closely-related OTUs in disjunct hosts and ranges is striking. For example, based on ITS sequences, a *Picea rubens* endophyte isolated in this study (NB-487-4H) is conspecific or closely related to endophytes from *Lycopodium annotinum* in Poland (JX981467; identities = 512/513 i.e. 99 %, gaps = 1/513), *Nothofagus solandri* in New Zealand (JN225881; identities = 511/512 i.e. 99 %, gaps = 0/513), and *Picea abies* in Finland (EF592102.1; identities = 508/511 i.e. 99 %, gaps = 0/511). NB236-1C, a *Picea mariana* needle endophyte closely related to *Mollisia nigrescens*, is conspecific with a *Vaccinium vitis-idaea* root endophyte isolate in northern China (KJ817299; identities = 779/779 i.e. 100%, gaps = 0/779). Six endophyte strains isolated from the stems of *Vaccinium angustifolium* and *V. corymbosum* in Eastern Canada share identical ITS sequences with NB236-1C (M. Sumarah, pers. comm.). Several GenBank ITS sequences representing a putative species (attributed variously as *Mollisia minutella*) were detected from a variety of hosts, host tissues, and locations, including *Picea abies* needles in the Czech Republic (FR837920), bog sediment in Russia (JX507688), *Ledum palustre* roots in China (KJ817294), *Picea abies* wood in Sweden (DQ008242), and *Pinus sylvestris* roots in Finland (KM068419).

The clade including *Barrenia* comprises several grass-inhabiting species such as *Barrenia panicia*, *Mollisia epitypha*, *Phialocephala bamuru*, and two distinct isolates named *Mollisia hydrophila* (CBS 233.71 and CBS 556.63) (Fig. 2.4). Many unnamed isolates or sequences also derive from grasses such as *Carex* spp., *Deschampsia flexuosa*, *Elymus mollis*, and *Saccharum* sp., although this clade also contains root isolates from Ericaceae (*Calluna vulgaris*, *Epacris pulchella*, *Vaccinium vitis-idaea*, *Woollisia pungens*), Orchidaceae (*Cymbidium insigne* and *Pseudorchis albida*), Pinaceae (*Picea abies*, *Pinus pinea*, *P. rigida*, and *P. sylvestris*), and other various host plants (e.g.: *Digitalis* sp., *Podophyllum peltatum*, *Vochysia divergens*). Several named apothecial species within this clade, such as *Mollisia epitypha*, *M. hydrophila*, *M. nigrescens* (NB-650), *M. obscura*, and *Tapesia villosa*, have well-developed melanized subicula, however this is probably a poor character for delineating above the species rank and may be even unstable within species (Aebi 1972). ITS sequences place *M. obscura* within the *Barrenia* clade in *Mollisia* s.l., its closest relative being an unidentified ericaceous (*Epacris microphylla*) root endophyte from Australia (AY268211.1; identities = 495/505 i.e. 98%, gaps = 2/505). *Mollisia obscura*, the type species of the synonymized mollisoid genus *Trichobelonium* (Aebi 1972, Richter and Baral 2008), is found on dead *Calluna* roots or stems and is morphological distinct, characterized by fusiform 3–7-septate ascospores and a well-developed dark subiculum (Fig. 2.23). Sequences in this clade originate from a wide geographic area, including North America, Northern and Central Europe, Australia, Brazil, and China.

Broad host and geographic ranges have been well-documented for the PAC root endophytes (Addy et al. 2000, Grünig et al. 2008b), although species in the PAC appear to be restricted to roots while other members of the lineage have been detected as endophytes in both roots and aboveground plant tissues. Besides root endophytes, species within the encompassing *Phialocephala* s.s. and PAC clade include endophyte isolates from cambium (*P. compacta*) and conifer needles (*P. amethystea*, *P. helena*, *P. nodosa*, *P. piceae*, *P. vermiculata*). *Phialocephala scopiformis* is an endophyte of *Picea* needles and cambium, suggesting that it systemically infects aboveground host tissues (Kowalski and Kehr 1995, Miller and Adams 2013, Tanney et al. 2016a). While *P. scopiformis* has apparently been reported only from *Picea*, a closely-related strain was isolated as a *Calluna vulgaris* leaf endophyte (FM200586.1; identities = 493/495 i.e.: 99%; gaps = 0/495). *Mollisia fusca* (CBS 234.71), isolated from apothecia on *Fagus sylvatica* in Switzerland, shares a similar ITS sequence with a *Phialocephala* strain isolated as a bark endophyte also from *Fagus sylvatica* in Switzerland (EU434850.1; identities = 476/481 i.e.: 99%; gaps = 2/481). *Phialocephala* and to a lesser extent *Mollisia* spp. are reported as endophytes of cambium, xylem, and bark in hardwood and softwood trees (Butin and Kowalski 1990, Kowalski 1991, Kowalski and Kehr 1992, Kowalski and Kehr 1995, Barklund and Kowalski 1996, Kowalski and Gajosek 1998). Kowalski and Kehr (1992) observed that many fungi, including *Phialocephala* and *Mollisia* strains, isolated from living tree branches were also the most frequent colonizers of dead branches, hypothesizing that this latent endophytic phase was associated with self-pruning.

The interactions between mollisioid endophytes and their plant hosts are not well understood. PAC root endophytes may exhibit mutualism, neutralism, or pathogenism with varying virulence, with such interactions apparently being strain-dependent and not correlated with species (Stoyke and Currah 1993, Vohník et al. 2005, Newsham 2011, Tellenbach et al. 2011, Tellenbach et al. 2012). The *Pinus rigida* root endophyte *Barrenia taeda* negatively affected root elongation in switchgrass (*Panicum virgatum*) seedlings, while the poaceae endophytes *B. panicia* and *Acidomelania panicicola* significantly increased root hair growth in switchgrass seedlings (Walsh et al. 2015). *Phialocephala subalpina*, a species in the PAC, reduced mortality and disease intensity caused by the oomycete pathogens *Phytophthora plurivora* and *Elongisporangium undulatum* (Tellenbach and Sieber 2012). A *Phialocephala sphaeroides* strain isolated as a *Picea abies* root endophyte inhibited in vitro growth of the plant pathogens *Botrytis cinerea*, *Heterobasidion parviporum*, and *Phytophthora pini*, increased root/shoot ratio of *Picea abies* seedlings, and prevented infection of seedlings by *H. parviporum* under in vitro conditions (Eeva et al. 2016). Conversely, *Phialocephala bamuru* was recently described as the causal agent of fairway patch, a serious emerging disease of golf course turfs in Australia that appears resistant to chemical control measures (Wong et al. 2015).

Many Mollisiaceae isolates produce secondary metabolites that may afford protection against plant pests and pathogens. A strain of the PAC species *Phialocephala europaea* produces secondary metabolites (sclerin and sclerotinin A) that significantly inhibit growth of the plant pathogen *Phytophthora citricola* (Tellenbach et al. 2012).

Sclerin and related compounds are phytotoxic to several cruciferous species in vivo, causing leaf chlorosis and necrosis (Pedras and Ahiahonu 2004). *Phialocephala scopiformis* isolates produce rugulosin, a bis-anthraquinone pigment first described from *Talaromyces rugulosus* (as *Penicillium rugulosum*) that exhibits antibacterial activity against *Streptococcus aureus* and moderate activity against *Globisporangium intermedium* (= *Pythium intermedium*) (Breen et al. 1955). A *P. scopiformis* strain inoculated in *Picea glauca* seedlings produces rugulosin in amounts deleterious to the eastern spruce budworm (*Choristoneura fumiferana*) (Sumarah et al. 2008a, Miller 2011). The fungus persists in the host over 10 years after inoculation and was reported to have spread to 40% of uninoculated seedlings in the lower canopy within three years (Miller et al. 2009). *Phialocephala vermiculata* (DAOM 229535), isolated as a *Picea glauca* needle endophyte, produces the macrocyclic antibiotic vermiculin and several natural products, including 6,7-dihydroxy-2-propyl-2,4-octadien-4-olide, that are toxic to spruce budworm cells (Findlay et al. 2003).

Two strains initially reported as *Phialocephala fortinii* isolated from rhizomes of *Podophyllum peltatum* produce podophyllotoxin, a lignan well-studied for its antiviral and antineoplastic properties (Eyberger et al. 2006). The podophyllotoxin-producing strain PPE7 was referred to as *Phialocephala podophylli* but awaits formal description (Arneaud and Porter 2015). A *Phialocephala* cf. *fortinii* root endophyte strain of *Rhodiola angusta* produces high yields of the bioactive tyrosols salidroside and *p*-tyrosol, which are normally harvested from *Rhodiola* tissues (Cui et al. 2016).

Production of podophyllotoxin and salidroside from *Podophyllum* and *Rhodiola* plant cell

and tissue cultures suggests the mutual production of these bioactive metabolites by both the plant host and endophyte, a controversial phenomenon exemplified by the supposed production of taxanes by *Taxus* endophytes (Stierle et al. 1993, Staniek et al. 2009, Heinig et al. 2013).

Tanney et al. (2016a) described connections between saprotrophic and endophytic *Phialocephala* species with mollisioid apothecia in the field. In this study, connections between unknown endophytes and field specimens include *Phialocephala amethystea*, *P. helena*, and *Mollisia novobrunswickia* detected as both needle endophytes and apothecia on decaying wood within the same forest stands. *Phialocephala helena* and *P. piceae* are closely related and morphological similar (oblong ascospores and strong yellow KOH reaction). Both species occur as *Picea* needle endophytes with apothecia often erumpent on fallen corticated hardwood branches (*Acer saccharum* and *Betula alleghaniensis*). An *Abies balsamea* endophyte (NB-238-1A) is conspecific with *Mollisia melaleuca* (CBS 589.84). Additional connections can be found in comparisons with unidentified GenBank sequence accessions; for example, *Mollisia nigrescens* has been isolated from apothecia on decaying wood in France (CBS 558.63) and Canada (NB-650) and as an endolichenic fungus of *Flavoparmelia caperata* in North Carolina (JQ761691.1; identities = 848/848 i.e. 100%, gaps = 0/848). A strain identified as *Mollisia olivascens* (CBS 293.59) shares an identical ITS sequence with the *Phialocephala urceolata* ex-type (UAMH 10827), isolated from commercial heparin solution (Wang et al. 2009). *Mollisia lividofusca* (CBS 231.71), isolated from apothecium occurring on *Lonicera caerulea* in Switzerland, shares similar ITS sequences with a *Picea*

abies needle endophyte from the Czech Republic (FR837926.1; identities = 785/787 i.e.: 99%; gaps = 1/787) and a *Picea glauca* needle endophyte from Canada (AY561210.1; identities = 595/595 i.e.: 100%; gaps = 0/595). A *Mollisia epitypha* herbarium specimen (DAOM 150777) collected from dead *Typha latifolia* stems in Ontario, Canada is closely related to the recently described Australian turf grass pathogen *Phialocephala bamuru* (KJ877190.1; identities = 501/507 i.e. 99%, gaps = 2/507). There is no described sexual state for *P. bamuru*; its relation to *M. epitypha* suggests searching for apothecia on dead culms of infected grasses.

Mollisia s.l. can no longer be regarded simply as saprotrophs associated with dead or decaying aboveground plant tissues (e.g.: Walsh et al. 2015). The life cycles of species within the lineage remain enigmatic; what role does endophytism play in the life cycles of endophytic species? Observations by Tanney et al. (2016a) show that endophytism in *Phialocephala scopiformis* and *P. piceae* is facultative and the fungus is not necessarily restricted to a specific host or host substrate. Endophytism in Mollisiaceae might represent a secondary life-history strategy that facilitates persistence and dispersal in the absence of primary substrates or in challenging environmental conditions, in the sense of the foraging ascomycete theory (Carroll 1999, Thomas et al. 2016). The ecological plasticity exhibited by these taxa is impressive and warrants additional attention and study.

Endophytism occurs throughout the lineage but is poorly studied outside of the PAC. The lack of available reference sequences and overall taxonomic neglect of

mollisoid taxa have resulted in the inability to effectively identify and categorize related endophytes. The genera *Acidomelania* and *Barrenia* were erected to describe root endophytes from Poaceae spp. and *Pinus rigida* in the New Jersey Pine Barrens (Walsh et al. 2014, Walsh et al. 2015). Based on the *RPB1* phylogeny, *Barrenia*, comprising two species, is polyphyletic and resides in a clade containing isolates of *Mollisia*, “*Tapesia*” (*Mollisia* conserved over the older name *Tapesia*; Hawksworth and David 1989), and the *Phialocephala hiberna* ex-type. In addition, *Phialocephala podophylli* is probably conspecific with *Barrenia taeda* based on ITS sequence similarity (KM042204.1 and KT598375.1; identities = 401/402 i.e.: 99%; gaps = 0/402). One of the rationales presented by Walsh et al. (2015) for erecting *Barrenia* was that its endophytic trophic mode distinguishes it from *Mollisia*. While there have been previous reports of *Mollisia* endophytes (Sieber 1989, Barklund and Kowalski 1996, Shamoun and Sieber 2000, Kowalski and Andruch 2012), endophytes in this lineage are usually reported as *Phialocephala* spp. because of a lack reference sequences, the absence of endophyte cultures linked to apothecial states, the preponderance of phialocephala-like asexual states in vitro, and lack of apothecia formed in vitro. In the absence of a working taxonomic framework, the taxonomic classification of endophytes within this lineage will continue to be uncertain, leading to reports of unidentified species or species placed in arbitrary genera or novel accommodative genera.

Divergent aquatic lineages

Phylogenetic studies using rDNA sequences report a close relationship between *Mollisia*, *Phialocephala*, and the aquatic genera *Loramyces* and *Vibrissea* (Wang et al. 2006a, Raja et al. 2008). This affiliation is unexpected because of considerable morphological differences with *Mollisia*, such as the long stipes of some *Vibrissea* spp., the perithecioid ascomata of *Loramyces*, and morphologically distinct ascospores of *Loramyces* and *Vibrissea* which are adapted to dispersal in aquatic environments. The LSU, *RPB1*, *TOP1* phylogenies strongly support the placement of *Vibrissea* outside the *Mollisia* lineage, while the other gene phylogenies place *Vibrissea* close to the *P. dimorphospora* s.s. clade or the PAC with variable support. Morphologically, it is more conceivable that *Vibrissea* is outside of the main lineage and that the discordance observed between the individual genes are a result of long-branch attraction artefacts and/or very highly conserved, less informative gene regions for the rDNA phylogenies; discrepancies between phylogenies using protein-coding genes (*RPB1*) and rDNA genes (SSU, LSU) are reported for example in Lecanoromycetes (Hofstetter et al. 2007). Evidence showing the placement of *Vibrissea* within Mollisiaceae based on the *LNS2* phylogeny is not compelling given the overall weakly-supported branches and discrepancies with other genes. Conversely, morphological dissimilarities between *Vibrissea* and other mollisioid taxa may not readily discount the placement of *Vibrissea* within Mollisiaceae; *Vibrissea* spp. possess paraphyses with refractive vacuolar bodies, exhibit anguillospora- and phialocephala-like asexual states, have a textura globulosa ectal excipulum comprised of pigmented, thin-walled, round cells, and some species

such as those previously placed within *Apostemidium* are sessile and more or less mollisioid. The divergent ascospore and ascus tip morphologies in *Vibrissea* may be phylogenetically-confounding autapomorphic characters resulting from adaptation to aquatic environments, similar to the divergent ascospore and ascomatal characters observed in *Loramyces* and *Obtectodiscus*. Also compelling is the association of *Anavirga dendromorpha* and its phialocephala-like synanamorph with *Vibrissea flavovirens* cultures flooded with water (as *Apostemidium torrenticola*; Hamad and Webster 1988). The phialocephala-like synanamorph exhibits dimorphic conidia; however conidia are often brown and may be rough, distinct from the typically hyaline and smooth conidia of *Phialocephala*. In this study, *Vibrissea flavovirens* CBS 121003 produced few sparsely-branched conidiophores bearing phialides similar to those described by Descals and Sutton (1976) when agar blocks containing mycelia were floated in sterile water. Additional taxon sampling with supplementary phylogenetically-informative genes to generate a more comprehensive phylogeny is required before the placement of *Vibrissea* can be confidently determined.

Loramyces, *Obtectodiscus aquaticus*, *Ombrophila hemiamyloidea*, and *Mollisia diesbacha* form a strongly supported clade within Mollisiaceae in all phylogenies except *LNS2*, which shows these species occupying a weakly-supported polytomous clade. All species except the basal *Mollisia diesbacha* are found in semi-aquatic or aquatic habitats; *Loramyces* and *Ob. aquaticus* occur on Poaceae spp. while *Om. hemiamyloidea* and *Mollisia diesbacha* are found on decaying hardwood. Semi-aquatic species within this clade exhibit remarkably deviating morphological characters from other mollisioid

taxa. *Loramyces* is characterized by perithecioid apothecia surrounded by gelatinous excipular hyphae and ascospores bearing gelatinous sheathes and long (100–140 µm in *L. macrosporus*) basal cellular appendages (Weston 1929, Ingold and Chapman 1952). The taxonomic placement of *Loramyces* was tentative, for example its assignment within the Sphaeriaceae, Trichosphaeriaceae, Hypocreales, and even its own monotypic family, Loramycetaceae (Digby and Goos 1987). *Obtectodiscus aquaticus* possesses more or less perithecioid apothecia with long filiform ascospores and was sampled in this study because of its resemblance to *Loramyces* spp. Baral (1999) noted several typical *Mollisia* characters present in *Ombrophila hemiamyloidea*, such as the textura globulosa ectal excipulum and refractive vacuolar bodies in the paraphyses that display a yellow KOH reaction, as well as features shared by *Niptera*, including similar spore morphology and the presence of a gelatinous ascospore sheath that turns red in IKI. Divergent characters ultimately led to its description in *Ombrophila*, namely a strongly gelatinized medullary excipulum and hyaline ectal excipulum. Verkley (2003) considered the apical apparatus and its reactivity with annular periodic acid (PA)-thiocarbohydrazide (TCH)-silver proteinate (PA-TCH-SP) more indicative of *Pezizula* rather than that *Vibrissea* and *Mollisia* relatives.

A Hysteronaevia scirpina (DAOM 147320) herbarium specimen is sister to *Obtectodiscus aquaticus* based on ITS sequences. There are 13 described *Hysteronaevia* spp., which are grammicolous, reported from terrestrial to partially-submerged or submerged substrates, and characterized morphologically by small 0.1–0.3(–0.8) mm diam apothecia immersed or erumpent on host culms or leaves, inamyloid asci,

generally large (12.5–)20–30(–40) × (1.5–)2–4(–8) μm, 0–1-septate, ellipsoidal to fusiform to subcylindrical ascospores, and sometimes a fimbriate margin (Nannfeldt 1984, Dennis and Spooner 1993, Shearer 1993, Raitviir 2008). Nannfeldt (1984) considered *Hysteronaevia* related to *Mollisia* (“Dermateaceae-Mollisioideae”), which is supported by the placement of the *H. scirpina* specimen within Mollisiaceae. Similar genera that have been classified in the *Dermateaceae* or considered relatives of *Mollisia*, such as *Diplonaevia*, *Hysteropezizella*, *Hysterostegiella*, *Laetinaevia*, *Micropeziza*, *Naevala*, and *Naeviopsis* are poorly represented by sequence data and may include species phylogenetically related to the *Loramyces* clade or other Mollisiaceae genera (Hein 1976, 1982). For example Hein (1976) described phialocephala-like conidiophores in cultures of *Naevala minutissima* and *Naeviopsis epilobii*. However, a collection of *Hysterostegiella typhae* (NB-638) is placed within Cenangiaceae based on ITS and LSU sequences (see chapter 4).

Mollisia diesbacha, a more or less typical mollisoid species, was collected from decaying hardwood in a terrestrial habitat and is basal to the semi-/aquatic species. Mollisoid ascomycetes are reported from submerged and/or partially submerged substrata (Fisher and Webster 1983, Shearer 1993). While an *Anguillospora* asexual state was described for *Loramyces juncicola*, no asexual states are attributed to *L. macrosporus*, *O. aquaticus*, or *O. hemiamyloidea*. In this study, phialocephala-like asexual states were observed for *Ombrophila hemiamyloidea* and *Mollisia diesbacha* after floating agar blocks containing mycelia for several weeks.

Asexual states have been induced in other aquatic mollisioid taxa by floating or flooding cultures with sterile water (Webster and Descals 1975, Descals and Sutton 1976, Fisher and Webster 1983, Digby and Goos 1987, Webster et al. 1993). It is unknown why this method induces sporulation in some Mollisiaceae species, e.g.: cues resulting from changes in exposure to ambient gases, increased moisture, exposure to a nutrient-poor substrate, being subjected to small movements while suspended in water (i.e.: thigmotropism), or the dilution of inhibitory factors that may otherwise accumulate in solid agar media. The induction of sporulation by means of mimicking aquatic conditions or observations of ingoldian conidia suggests a larger diversity of aquatic *Mollisiaceae* species than perhaps realized. However, these reported synanamorphs should be investigated further. For example, the classification of the asexual state of *Anguillospora crassa* in *Mollisia* is somewhat dubious based on the hyphal elements composing the excipulum as described by Webster (1961) and the placement of purported *A. crassa* and *A. furtive* GenBank sequences in *Hymenoscyphus* s.l. (*Cudoniella* or *Phaeohelotium*) (Qiao et al. 2015). An *Anguillospora crassa* specimen (NB-681) occurring on wet wood was cultured by J.B. Tanney and yielded both *Anguillospora* and phialidic synanamorphs on CMA (Fig. 2.24). The phialidic synanamorph was characterized by ampulliform phialides with deep, funnel-shaped collarettes, which developed from sparingly-branched penicillate conidiophores or directly from *Anguillospora* conidia (microcyclic conidiation). This synanamorph superficially resembles *Phialocephala*; however ITS sequences place this isolate in *Hymenoscyphus* s.l. Additionally, J.B. Tanney made several collections of a species

characterized by sessile mollisioid apothecia from wood partially submerged in stream water (Fig. 2.25). Apothecia were remarkably similar to *Mollisia*, including paraphyses with refractive vacuole bodies and a thick medullary excipulum often found in *Mollisia* spp. from wet environments, although the inamyloid asci contradicted this initial identification. ITS sequences place this species in *Hymenoscyphus* s.l., closely related to *Anguillospora crassa* (identities = 542/544 i.e. 99 %, gaps = 0/544), and morphologically it resembles a sessile species within the *Hymenoscyphus* cf. *imberbis* group. The *Hymenoscyphus* cf. *imberbis* ascospores were similar to the *Mollisia* state of *A. crassa* described by Webster (1961), (7–)7.5–10(–11) × 3–4 µm vs. 7.5–10 × 2.5–3 µm, and younger ascospores also contained two prominent bipolar guttules similar to those of *A. crassa*. Differences such as hymenium colour (white or cream to pale blue vs. white to cream) and apothecium diameter (1.5–2 mm vs. 1 mm) may be a result of the *A. crassa* state developing in vitro. These observations suggest that Webster (1961) mistook the *A. crassa* sexual state, probably a mollisioid *Hymenoscyphus* s.l. species, for a *Mollisia* species.

Casaresia sphagnicola is an aquatic hyphomycete that produces impressive dematiaceous stauroconidia and is reported to have a *Phialocephala* synanamorph and *Mollisia* sexual state (Webster and Descals 1975, Webster et al. 1993). Repeated attempts by the first author to culture *Casaresia sphagnicola* from conidia failed, possibly due to robust conidia remaining intact past viability, however the phylogeny of this distinct species should be explored.

ITS and LSU sequences of the ex-type culture placed *Variocladium giganteum*, an aquatic hyphomycete, in Mollisiaceae (Baschien et al. 2013); however, the *RPB1* phylogeny suggests this placement is misleading and a result of incomplete taxa sampling causing long-branch attraction. However, convincing phialocephala-like asexual states have been reported in cultures of *V. giganteum* and *V. rangiferinum* (Willoughby and Minshall 1975, Descals and Webster 1982). Sequences are not available for *V. rangiferinum*, which could conceivably belong in Mollisiaceae. Alternatively, it is possible that purported *Variocladium* cultures forming phialocephala-like synanamorphs were misidentified and are undescribed aquatic species related to Mollisiaceae. The placement of *Strossmayeria basitricha* within Mollisiaceae based on a LSU phylogeny by Hustad and Miller (2011) is also dubious because of branch length in the *RPB1* phylogeny. *Strossmayeria basitricha* produces two synanamorphs: a pseudopspiropes dematiaceous asexual state dissimilar to those of mollisoid species and a phialidic asexual state with flaring collarettes reminiscent of *Phialocephala*, however unlike *Phialocephala* the phialides are produced directly from ascospores and asci (and paraphyses according to Iturriaga and Korf 1990) (Fig. 2.26).

The ITS phylogeny depicts a strongly-supported clade of semi-aquatic, lignicolous species distinct from the *Loramyces* clade (Fig. 2.4). This clade comprises herbarium specimens identified as *Niptera discolor* (Fig. 2.27), *Niptera ramincola* (Fig. 2.28) and *Mollisia caesia* (= *Niptera caesia*) (Fig. 2.29), a GenBank accessioned sequence identified as *Mollisia ventosa*, and a *Niptera* sp. collected from a decaying branch in a drying stream in New Brunswick (NB-714). The ITS phylogeny places the *Niptera* clade sister to

Vibrissea and the PAC while the *RPB1* phylogeny strongly supports (P.P. = 0.99) the placement of *Niptera* sp. (NB-714) basal to the *Phialocephala* s.s. clade and *Mollisia ligni* var. *olivascens* (CBS 291.59) and *Phialocephala vermiculata* (Fig. 2.8). These species are generally characterized by fusiform, 0–1(–3)-septate ascospores, long asci with narrow and elongated apical pores, and frequently well-developed and melanized subicula. Dennis (1972) noted that while *Niptera* was an exceptionally well-defined genus for its day (Fries 1849), interpretations by other authors obscured the generic concept by including terrestrial species that would be referred to *Mollisia* or similar genera and not congeneric with the type species *N. lacustris* on the basis of 1-septate ascospores (De Notaris 1864, Rehm 1891). Consequently, the name *Niptera* has been applied to over 150 species, including unrelated species such as *Lophodermium arundinaceum*, and species have been transferred from *Niptera* to *Mollisia* and other genera such as *Belonium*, *Belonopsis*, *Nimbomollisia*, and *Scutomollisia*. Given the nomenclatural priority of *Niptera* (1849) over *Mollisia* (1871) and the existence of a coherent *Niptera* clade that shares some morphological and ecological characters, as suggested in this study, the segregation of a *Niptera* s.s. clade within Mollisiaceae is feasible. It is also conceivable that *Niptera* s.l. comprises several phylogenetically distinct clades sharing morphological homoplasies resulting from adaptation to semi-aquatic habitats. However, the phylogenetic placement of *Niptera lacustris* must first be resolved and additional sequencing of *Niptera* spp. is required.

Morphological characters including gelatinous sheaths and septation of ascospores have been used to delineate semi-aquatic mollisioid genera such as

Nimbomollisia and *Niptera* (Nannfeldt 1983). However, these genera remain mostly unsequenced and their relationship with *Mollisia* s.l. and the phylogenetic resolution of these morphological characters remains unknown. Collecting, culturing, and sequencing unrepresented mollisioid species or attributed aquatic synanamorphs from semi-aquatic habitats will fill in taxonomic gaps and provide insight into the evolution of aquatic-adapted species and genera throughout the lineage.

***Phialocephala* s.s.**

Phialocephala contains species occurring throughout Mollisiaceae (e.g.: *P. hiberna* and *P. scopiformis*) and in other classes (e.g.: *P. fluminis* and *P. virens*; Day et al. 2012). *Phialocephala* s.s., defined by the phylogenetic placement of the type species *P. dimorphospora*, includes *P. aylmerensis*, *P. biguttulata*, *P. botulispora*, *P. catenospora*, *P. collarifera*, *P. dimorphospora*, *P. lagerbergii*, *P. mallochii*, *P. nodosa*, *P. oblonga*, *P. repens*, and *Mollisia heterosperma*. *Mollisia heterosperma* is therefore transferred to *Phialocephala* above and the authenticated strain CBS 292.59 selected as an ex-epitype strain. *Phialocephala* s.s. according to Tanney et al. (2016a) is almost exclusively comprised of lignicolous saprotrophs except for reports of *Phialocephala nodosa* isolated as a foliar endophyte from *Picea mariana* and *Pinus strobus* in Canada (Tanney et al. 2016a) and *Populus euphratica* in China (FR865028). Several dematiaceous synanamorphs are associated with species of *Phialocephala* s.s., including the diplococcium-like asexual state of *P. catenospora* and a synnematosous asexual state formerly placed in *Paradidymobotryum* (*P. oblonga*) (Tanney et al. 2016a). Sexual states

attributed to *Phialocephala* s.s. are typically greyish brown or greyish blue in color with ellipsoidal to oblong ascospores within the range of (7–)8–10(–12) × (2.5–)3–4 μm. In this study, apothecial and asexual state collections representing two distinct species were collected from decaying wood in Canada and described as *Phialocephala biguttulata* and *P. collarifera*. Unidentified GenBank ITS sequences show several putative undescribed species within *Phialocephala* s.s. (Fig. 2.5).

***Mollisia* s.s.**

The delineation of *Mollisia* s.s. is dependent on the epitypification of the type species *M. cinerea*. Current efforts are underway to designate an epitype collected close to the type locale in Jena, Germany (A. Gminder pers. comm.). Based on the *RPB1* phylogeny and preliminary data, *Mollisia* s.s. is probably sister to a clade comprising *Acidomelania*, *Mollisia melaleuca*, *M. novobrunswickia*, *M. prismatica*, and related conifer needle endophytes. Unexpectedly, these clades are sister to the *Loramycetes-Obtectodiscus-Ombrophila hemiamyloidea* semi-aquatic clade.

Nipterella

Starbäck (1895) described *Niptera duplex* from juniper wood and while the author noted that the species probably warranted its own genus (“Sie scheint mir sogar zu verdienen, als eine besondere Gattung *Nipterella* unterschieden zu werden”), he did not formally describe *Nipterella*. Dennis (1962) later validated *Nipterella* to accommodate *N. duplex*, the type species, and *N. parksii*, formerly *Belonidium parksii* (as “*Belonidium parksii*” Cash 1936). The third *Nipterella* species, *N. tsugae*, occurs on self-

pruned branches of *Tsuga heterophylla* (Funk 1978). The three species share some morphological characters including hymenia ranging from yellow to bluish green in colour and an inrolled margin composed of dark moniliform cells. Dennis (1962) considered *Nipterella* as a genus within the Helotiaceae subfamily Encoelioideae, distinguished from other genera such as *Encoelia* by the septate ascospores and amyloid asci. Müller and Defago (1967) considered *Nipterella* in the Dermateaceae and synonymized it with *Dibeloniella* while Korf (1973) referred to it within the Helotiaceae-Encoelioideae. Starbäck (1895) noted that the ectal excipulum of *N. duplex* is similar to that of *Mollisia*.

A specimen of *Nipterella parksii* was available for study. *Nipterella parksii* (DAOM 56610) is characterized by a greenish yellow to greyish yellow (1A5-1B8) hymenium, darkly pigmented (10F3) ectal excipulum composed of moniliform cells, giving the apothecia a scurfy appearance, an inrolled and plicate margin comprised of cylindrical moniliform, scale-like marginal cells, and 3-septate, fusiform-ellipsoidal ascospores (Fig. 2.30). The ITS sequence generated from this specimen place it with weak support (P.P. = 0.63) within a polytomous group containing *Mollisia lividofusca* (CBS 231.71), *Tapesia fusca* (CBS 555.63), "*Tapesia cf. fusca*" (NB-545, NB-610), *Phialocephala sphaeroides*, *Mollisia prismatica*, and cultures attributed to *M. caesia* (CBS 220.56), *M. discolor* (CBS 289.59), *M. fallens* (CBS 221.56), and *M. ventosa* (CBS 322.77). While *Nipterella parksii* is clearly in Mollisiaceae, its precise placement is unknown. An NCBI BLAST search yields a closest match with purported *Mollisia minutella* strains (e.g.: KJ817294; identities = 415/443 i.e. 94 %, gaps = 6/443).

Family placement of Mollisia and related genera

Mollisia has been variously placed within Dermateaceae or Mollisiaceae (Rehm 1891 [as "Mollisieae"], Velenovsky 1934, Kirk et al. 2008, Pärtel et al. 2016), with the consensus being to refer to it within Dermateaceae (e.g.: Index Fungorum [<http://www.IndexFungorum.org>], MycoBank [<http://www.MycoBank.org>], NCBI taxonomy [<http://www.ncbi.nlm.nih.gov/taxonomy>]). *Mollisia* and allied taxa clearly do not belong in Dermateaceae and should be considered in Mollisiaceae (Goodwin 2002). *Phialocephala*, considered a member of Vibrisseaceae, is now recognized within Mollisiaceae. Vibrisseaceae is considered distinct from Mollisiaceae based on *RPB1* and *TOP1* phylogenies and requires a modern circumscription (e.g.: polyphyletic due to inclusion of *Chlorovibrissea* and *Myxocephala*; Sandoval-Leiva et al. 2014, Nonaka et al. 2015). *Loramyces* is strongly supported within Mollisiaceae and recognition of Loramycetaceae would result in a paraphyletic Mollisiaceae; therefore, Loramycetaceae is synonymized with Mollisiaceae to maintain monophyly and to better describe the taxonomy of *Loramyces* and related taxa.

Taxonomic issues and solutions

One of the original goals of this study was to generate phylogenetic data and make taxonomic changes accordingly to promote taxonomic stability and practicality in this lineage. It soon became evident that that such action would be premature and likely initiate a turbulent taxonomic phase resulting in ephemeral name changes and more confusion for taxonomists and users alike. In this respect, this study may serve as

a prodromus for a revision of Mollisiaceae. Sampling is too inadequate to enable meaningful taxonomic decisions at the present time. While previous workers depending solely on morphological characters could not make sufficient progress because of confounding characters, DNA sequence-based methods now facilitate rapid species delineation, identification, and phylogenetic reconstruction. For the first time in mycological history, the future of *Mollisia* taxonomy is optimistic.

Major issues that challenge users and the development of a stable and informative taxonomic framework in an immediate timeframe include: (1) the identification of unknown OTUs within a lineage spanning many poorly-characterized and unsequenced genera; (2) the lack of reference sequences from type or epitype material; (3) the description of novel species (or genera) in the absence of proper study of historical species concepts; and (4) the nomenclatural and taxonomic issues surrounding this entangled lineage.

Detection and identification of fungal OTUs is becoming more reliant on DNA sequence-based methods. Without authenticated reference sequences, preferably originating from type or epitype material, users will be unable to confidently identify related OTUs in their research or work. In biodiversity and ecology studies, the inability to assign specific or taxonomic qualifiers significantly diminishes insight into biological systems and reduces the overall value of data generated from next generation sequencing (NGS) technology. Populating sequence databases with reference sequences will provide taxonomic benchmarks for users, increasing taxonomic

resolution. However, effectively generating reference sequences requires concerted sampling efforts from new collections and authenticated and type specimens.

Unlike other genera such as *Penicillium*, few authenticated or ex-type cultures of mollisoid species exist because previous workers did not prioritize culturing specimens or accessioning strains in culture collections. Consequently, authenticated specimens are restricted primarily to herbarium specimens, with few ex-type cultures available. Amplification of DNA from herbarium specimens can be challenging because of DNA degradation resulting naturally or accelerated from post-collection practices, while contaminating or co-occurring fungi can result in amplification of non-target DNA. The latter issue can be at least partly solved by developing taxon-specific primers, however this may be infeasible in cases where the phylogenetic placement of the targeted fungus is unknown and reference sequences to assist in primer design are unavailable. In some cases, sequencing attempts may be impermissible because of specimens being in too poor condition, insubstantial, or even lost.

Epitypification with new collections offers an alternative to destructive sampling of type specimens and can provide additional information such as detailed morphological observations including vital taxonomic characters (Baral 1992, Van Nooren 2010). New collections can be used to generate cultures, which may in turn provide accessional material for inoculation studies, characterization of secondary metabolites, in vitro studies, whole genome sequencing, etc. Careful and proper epitypification of described *Mollisia* and allied taxa is encouraged, however the large

number of insufficiently described or apparently morphologically indistinct species is problematic. A pragmatic strategy must be adopted to assist progress: sequence directly from type or authenticated material when possible, prioritize the epitypification of important or morphologically distinct species, and encourage the description of novel species. The high quality description of novel species from apothecia or cultures (sterile or sporulating) will provide a wealth of data associated with reference sequences. The risk of describing a previously named species is real; however propelling Mollisiaceae taxonomy into the 21st century at the expense of some novel species eventually being synonymized is a necessary risk. Hibbett et al. (2011) estimated the odds of an unidentified molecular operational taxonomic unit (MOTU) representing a species that is described but lacking reference sequences in GenBank as 18:1 or 44:1, depending on global biodiversity estimates. Taking into account these estimates of error, the historical difficulty in discerning *Mollisia* species from one another, lack of recent taxonomic work, and paucity of global surveying of Mollisiaceae species, it becomes evident that the description of novel species should be accelerated and not delayed because of concern for unintentionally redescribing named species. Additionally, unidentified sequences annotated with collection and morphological data can be shared and integrated into a repository, facilitating the incremental understanding of species concepts worldwide (Hosoya et al. 2015).

The polyphyletic nature of most genera presented in the phylogenies in this study presents a dilemma when describing novel species. What generic name should one choose? Several nomenclatural and taxonomic options will present themselves in

the future: (1) the entire lineage is transferred to *Mollisia* in an effort to create a large all-encompassing generic concept (“lumping”); (2) the bulk of mollisioid taxa are considered *Mollisia* and morphologically divergent taxa are maintained in distinct genera, regardless of monophyly; and (3) the lineage is divided and genera are erected or maintained based principally on monophyly (“splitting”).

The first option simplifies taxonomic decisions by favouring a large generic concept based on the morphotaxonomic concept of *Mollisia* s.l. Upholding a large *Mollisia* generic concept acknowledges the history of the genus, the nomenclatural priority of *Mollisia* over most genera within the lineage (although not all, e.g.: *Trimmatostroma* 1837, *Niptera* 1849, *Cheirospora* 1850), the number of species attributed to *Mollisia* versus other genera, and the typical mollisioid apothecial morphology found throughout the lineage. Consequently, the aquatic *Loramycetes*-*Obtectodiscus*-*Ombrophila hemiamyloidea* clade would be transferred to *Mollisia* to maintain a monophyletic generic concept. This transfer would weaken the morphotaxonomic concept of *Mollisia* and cause a loss of information, albeit in a small group of somewhat obscure species. Morphologically-distinct species, such as those placed in *Niptera* and other mollisioid genera, are undersampled, poorly represented by DNA sequences, and could conceivably form monophyletic groupings supported by morphological, biological, and ecological similarities. Similarly, additional sampling may support the recognition of patterns, such as the existence of clades containing more host-, biogeographic-, or substrate-specific species that share corresponding life histories. Introducing or maintaining genera to accommodate such clades may be more

informative than lumping them under the name *Mollisia*. A concession might be the eventual recognition of informal sections in *Mollisia* in the sense of *Agaricus* or *Trichoderma*, although the use of such sections in Mollisiaceae may be uninformative for non-taxonomists. Considering the current taxonomic confusion and relatively few users, relegating the name *Mollisia* to a moderate-sized clade would not result in significant inconvenience. For example, most field mycologists are unable to confidently identify *Mollisia* spp. using morphological characters; therefore, using "mollisia" as an informal descriptor for mollisioid apothecia would equally suffice.

Furthermore, while accepting a broad *Mollisia* generic concept may be more convenient in the short term, it is likely to be unstable, less informative in conveying biological and ecological traits for monophyletic groups (e.g.: *Loramyces* and *Phialocephala* s.s.), and its adoption is premature based on the lack of sampling. From a pragmatic standpoint, research within this lineage is primarily focused on species classified within *Phialocephala* s.l. (e.g.: the PAC), therefore this option would result in name changes that would impact users. While *Phialocephala* s.l. is polyphyletic, with species occurring not only throughout Mollisiaceae but also in distant classes (Grünig et al. 2002, Grünig et al. 2009), *Phialocephala* s.s. is clearly defined based on the recent epitypification of the type species *P. dimorphospora* (Tanney et al. 2016a). This clade is comprised of species primarily isolated from wood that exhibit dematiaceous synanamorphs previously unattributed to *Phialocephala*. While the identity of *M. cinerea* s.s. has been speculated upon for some time (e.g.: Crossland 1896), this question still cannot be sufficiently answered because of a lack of discerning

morphological characters garnered from descriptions of the type specimen (Batsch 1786, Karsten 1871, Dennis 1950) and, more unfortunate, the reported loss of the type specimen (A. Gminder, pers. comm.). Relegating the strongly supported *Phialocephala* s.s. concept to *Mollisia*, a genus typified by an ambiguous species lacking a type specimen and of unknown phylogenetic placement, is unwise. However, it must be noted that isolates identified as *Cheirospora botryospora* and *Niptera* sp. are phylogenetically placed within the *Phialocephala dimorphospora*-PAC clade, creating potential nomenclatural issues because of priority. Efforts to epitypify *M. cinerea* are underway (A. Gminder, pers. comm.) and crucial for understanding generic boundaries.

The classic mollisioid apothecium may prove to be a less significant character for generic delineation; therefore maintaining a large *Mollisia* genus based on the occurrence of uninformative homoplastic characters throughout the lineage would be misleading. For example, mycosphaerella-like sexual states are present in ca. 50 genera occurring across several families in the Capnodiales (Dothideomycetes) (Crous 2009, Crous et al. 2009a, Crous and Groenewald 2013); upholding a taxonomic system that emphasizes highly conserved sexual state morphological characters over other evidence (e.g.: molecular, asexual state morphology) would be deceiving and result in less informative generic concepts. The historic inability to reliably identify and classify mollisioid taxa based on apothecial morphology may indicate the limits of taxonomic informativeness for these characters across the lineage.

Alternatively, asexual state morphology may provide more taxonomic insight, for example as seen in the asexual states of *Calonectria*, Mycosphaerellaceae, and Teratosphaeriaceae (Lombard et al. 2010) (P. Crous, pers. comm.). Cultural studies of *Mollisia* are uncommon and an apothecium-centric approach has resulted in the neglect of potentially taxonomically informative asexual state characters (but see: Le Gal and Mangenot 1956, 1961, Aebi 1972, Tanney et al. 2016a). Tanney et al. (2016a) described dematiaceous synanamorphs in *Phialocephala* s.s. that could be used to readily distinguish known species, including microsclerotia composed of moniliform cells (*P. nodosa*), diplococcium-like chains of didymo- and phragmoconidia (*P. catenospora*), and didymoconidia produce in short acropetal chains from tall (up to 120 µm) synnemata (*P. oblonga*). Crous et al. (2015c) reported an unexpected connection between *Phialocephala* and *Cheirospora botryospora*, a fungus commonly found on dead branches of *Fagus* spp. *Cheirospora botryospora* produces bulbils composed of globose cells surrounded in a gelatinous sheath from acervuli erumpent through the host bark; on OA media, *C. botryospora* produces bulbils and a phialocephala asexual state. ITS and LSU sequences place *C. botryospora* close to *Phialocephala piceae* and *P. helena* within the PAC and *Phialocephala* s.s. clade. The description of distinctive synanamorphs throughout Mollisiaceae may provide important ecological and taxonomic information and warrant further study to assess whether such morphological characters provide resolution to species or clade (Table 2.1).

The second option compromises by encompassing most species within a large *Mollisia* genus and accepting select distinct species or clades as polyphyletic or

paraphyletic groups. For example, most species in the lineage presented in the large *RPB1* phylogeny (Fig. 2.8) would be considered *Mollisia* except for the divergent *Loramycetes* clade and perhaps the PAC and *P. dimorphospora* s.s. clade. The consensus among most taxonomists is that proposing paraphyletic and polyphyletic genera is unacceptable (Vellinga et al. 2015). As sampling increases and patterns emerge, this option would eventually capitulate to the division of a broad *Mollisia* genus, favouring more informative and monophyletic generic concepts.

The third option, to divide the lineage into genera based primarily on monophyly now inferred from molecular sequences, is likely to be favoured. Increased sampling and a polyphasic taxonomic approach combining characters including molecular phylogenetic data, morphology, physiological, biology, ecology, etc. will lead to more informative and elegant generic concepts within Mollisiaceae. Phylogenetic-based taxonomic systems may prove impractical for users relying entirely on morphology for identification. However, given the overall paucity of work being conducted on mollisioid discomycetes and the significant challenges imposed by the current taxonomic system regarding identification and classification, such criticism hardly warrants the maintenance of an artificial taxonomic system under the guise of serving the relatively few users who actively collect mollisioid specimens. A taxonomy based on useful phylogenetic concepts and adopted by endophyte researchers and other users will encourage the accumulation of relevant data and cumulatively improve our understanding of Mollisiaceae biodiversity and ecology. This third option requires effort and time for effective development; if genera are erected in a piecemeal fashion based

on current data, the results may be a taxonomic system characterized by many small, uninformative, and most likely unstable genera. Waiting for complete sampling of Mollisiaceae before taking action is infeasible and impractical; therefore, taxonomists must exercise due diligence when making taxonomic changes in light of the large sampling gaps and known taxonomic issues. Epitypification of *M. cinerea* and type species of other mollisioid genera is the first critical step to delineating generic concepts and building a strong taxonomic framework. Notably, the online database MolliBase was recently conceived to facilitate the connection of *Mollisia* sequences with apothecial morphology and collection data (Hosoya et al. 2015).

While waiting for additional data is prudent, some direction is required to assist users. In this study, the need to describe novel endophyte and/or apothecial isolates is emphasized. Deciding in which genus to describe novel species was challenging in light of the taxonomic shortcomings and risk of taxonomic instability. A parsimonious approach was chosen: species occurring within the *P. dimorphospora* s.s. and PAC lineage were described in *Phialocephala* and species outside of this lineage were described in *Mollisia*. These generic designations are admittedly provisional but are probably the most pragmatic course of action. Until major taxonomic issues are addressed and sampling is adequate, the haphazard or unwarranted erection of novel genera within this lineage should be avoided in the pursuit of taxonomic stability and informativeness, or proceed only when sufficient supportive evidence exists, i.e.: a monophyletic clade comprising several species sharing distinct characters. A taxonomic upheaval in this lineage is likely in the horizon; being parsimonious with name changes

and mindful of guidelines for introducing new genera presented by Vellinga et al. (2015) will ensure a more stable, user-friendly taxonomic system.

The occurrence of enigmatic endophytes, pathogens, and producers of interesting secondary metabolites within this lineage of largely forgotten or ignored taxa offers exciting research opportunities not limited to taxonomy, ecology, evolutionary biology, comparative genomics, natural products chemistry, plant-fungus interactions, and biological control. While Mollisiaceae has been a long-neglected and even maligned family, the application of DNA sequence-based identification and phylogenetic methods allow for more accurate species delineation and progress towards an effective taxonomic framework, facilitating further research and communication of taxa. The major obstacles to this progress are a lack of participating taxonomists and a dearth of sampling. The latter can be ameliorated through the active characterization and sequencing of specimens from fresh field collections, culture collections, and herbaria. The convergence of evidence indicates that Mollisiaceae species are much more important than previously realized and offer stimulating and pragmatic research opportunities.

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AUTHOR CONTRIBUTIONS

JBT conceived and designed the work. JBT acquired the data, provided strains, collections, and sequences from Canadian material; JBT provided the species descriptions, tested all markers and generated all sequences, conducted cultural studies and phylogenetic analyses, deposited cultures in DAOMC and specimens in DAOM. JBT analyzed and interpreted the data and JBT and KAS interpreted taxonomic considerations. JBT drafted the article; JBT and KAS critically revised the manuscript for content.

**Chapter 3: Production of antifungal and antiinsectan
metabolites by the *Picea* endophyte *Diaporthe maritima*
sp. nov.**

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ABSTRACT

The genus *Diaporthe* comprises close to 800 species, with around 2,000 names attributed to it and its asexual morphs previously recognized in *Phomopsis*. *Diaporthe* species are common plant associates, including saprotrophs, pathogens, and endophytes affiliated with a diverse range of hosts worldwide. In this study, an unknown *Diaporthe* sp. belonging to the *Diaporthe eres* species complex was frequently isolated as an endophyte from healthy surface-sterilized *Picea mariana* and *P. rubens* needles in the Acadian forest of Eastern Canada. Morphological observations and the application of the genealogical concordance phylogenetic species recognition concept using four unlinked loci (ITS, *Apn2*, *TEF1 α* , and *BenA*) support the distinctiveness of this species, described here as *Diaporthe maritima*. The crude liquid culture extracts from this new species showed potent antifungal activity towards the biotrophic pathogen *Microbotryum violaceum* in a screening assay necessitating an investigation of its natural products. Three dihydropyrones, phomopsolides A (1), B (2) and C (3), and a stable alpha-pyrone (4), were characterized by mass spectrometry and spectroscopic techniques. All isolated metabolites individually demonstrated in vitro antifungal and antibiotic activity towards the Gram-positive bacterium *Bacillus subtilis*. The Acadian Forest Region of Eastern Canada has proven itself to be a rich source of biodiversity that has the potential to deliver environmentally sustainable pest management practises.

KEYWORDS

foliar endophytes; phylogenetic species; GCPSR; Diaporthales; natural products;

phomopsolides

INTRODUCTION

Diaporthe species are among the most frequently isolated endophytes of various above-ground plant tissues (Toti et al. 1993, Murali et al. 2006, Vieira et al. 2014, Tateno et al. 2015). Endophytes operate along the endosymbiont-pathogen continuum, with reports of species or strains providing conditionally mutualistic benefits to the host such as drought-tolerance or protection from pathogens or pests (Gange et al. 2012, Hubbard et al. 2014). Foliar endophytes of conifer trees limit the impact of host pests by the production of antiinsectan (Findlay et al. 2003, Sumarah et al. 2005, Sumarah and Miller 2009) and antifungal (Sumarah et al. 2011, McMullin et al. 2015, Richardson et al. 2015) metabolites. The positive effect of endophytic infection is illustrated by the *in planta* production of the dimeric anthraquinone rugulosin by *Phialocephala scopiformis*, a needle endophyte of *Picea* spp., which delays the larval development of the destructive pest *Choristoneura fumiferana* (Eastern spruce budworm) (Miller et al. 2008). Previous investigations into the mycological and chemical diversity of foliar endophytes in the Acadian Forest Region have resulted in the discovery of novel biologically active metabolites from unidentified or undescribed fungi (Sumarah et al. 2008b, Sumarah et al. 2010, Richardson et al. 2015).

The genus *Diaporthe* includes saprotrophic, endophytic and pathogenic species associated with a wide variety of vascular plant hosts worldwide. It comprises nearly 800 described species, disregarding the >950 species or names attributed to its asexual state (formerly *Phomopsis*; Uecker 1988, Rossman et al. 2015). Species identification is often hindered by morphological characters that are little differentiated and often

overlap and by a dearth of reliable reference sequences. Previously, species were often defined based on the host or substratum, but molecular studies show that while some species are host-specific, many have wide host ranges and broad distributions (Wehmeyer 1933, Rehner and Uecker 1994, Udayanga et al. 2012). The existence of large phylogenetic species complexes further complicates the identification and delineation of *Diaporthe* species. The delineation of species within complexes is important, because it provides insight into speciation and geographic range, which assists in the detection and control of important pathogenic species. Recently, epitypification efforts and the application of the genealogical concordance phylogenetic species recognition concept (GCPSR; Taylor et al. 2000) using multiple unlinked loci have proved useful in distinguishing phylogenetic species within *Diaporthe* complexes, including *D. eres* (Udayanga et al. 2014b) and *D. sojae* (Udayanga et al. 2015).

A survey of endophytic fungi isolated from healthy surface-sterilized *P. mariana* and *P. rubens* needles in the Acadian forest of Eastern Canada resulted in the frequent recovery of a species belonging to the *Diaporthe eres* species complex (DESC) that yielded a crude liquid culture extract that elicited antimicrobial activity. This species was connected with field specimens sporulating on dead or dying *Picea* branch tips. In this chapter, the novel species *D. maritima* is described, supported by morphological observations and multilocus phylogenetic analyses, and its bioactive natural products are investigated.

MATERIALS AND METHODS

Sampling and isolation of fungi

Branches of *Picea mariana* and *P. rubens* of various age classes were collected in New Brunswick, Canada and stored in plastic bags at 4 °C. Endophytic fungi were isolated and subcultured from surface-sterilized needles following the methods of Tanney et al. (2016a).

Field specimens with phomopsis-like pycnidia were collected and conidia from cirrhi were streaked on 2% malt extract plates (MEA; 20 g Bacto malt extract, Difco Laboratories, Sparks, MD, U.S.A.; 15 g agar, EMD Chemicals Inc., Gibbstown, NJ, U.S.A; 1 L distilled water) and incubated in the dark at 16 °C. Resulting cultures were inoculated on various media including cornmeal agar (CMA; Acumedia Manufacturers Inc., Lansing, MI, U.S.A.), oatmeal agar (OA; Samson et al. 2004), spruce needle potato agar (SNPA; modified from Su et al. 2012), MEA, and 1.5% water agar (WA, with 1 mL trace metal solution; Crous et al. 2009b) with or without the addition of sterile *Picea* needles on the agar surface. These cultures were incubated at 16 °C or 20 °C under a 12/12 light cycle or at room temperature exposed to ambient light in an attempt to induce the formation of ascomata and conidiomata. To determine cardinal temperatures, select strains (NB-365-71I, NB-382-2E, NB-464-3A) were inoculated onto 9 cm MEA plates and incubated at 5 °C intervals from 5–40 °C; each treatment was repeated in triplicate.

Morphological studies

Vertical sections of pycnidia from cultures and field specimens were cut by hand or with a freezing microtome and for microscopy were mounted in either water, 85%

lactic acid, or 5% KOH. Microscopic measurements were taken from material mounted in water and are presented as ranges calculated from the mean \pm standard deviation of each measured value, with outliers in brackets. Colony colours were described using the alphanumeric codes of Kornerup and Wanscher (1978). Observations were made using an Olympus BX50 light microscope (Olympus Optical Co., Tokyo, Japan) and micrographs were captured using an Evolution MP Color Camera (Media Cybernetics, Silver Spring, CA, U.S.A.) and Image-Pro Plus v6.0 (Media Cybernetics) or InfinityX-32 camera (Lumenera Corp., Ottawa, ON, Canada) and Infinity Analyze v6.5.2 (Lumenera Corp.) software. Colony macrophotographs were captured with a Nikon Coolpix P5000 (Nikon Inc., Tokyo, Japan) and photographic plates were assembled using Adobe Photoshop v5.5 (Adobe Systems, San Jose, CA, U.S.A.).

Phylogenetic studies

Total genomic DNA was extracted from 4–12-wk old cultures using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, U.S.A.) following the manufacturer's protocol. The primers ITS4 and ITS5 (White et al. 1990) were used to amplify and sequence the nuc internal transcribed spacer rDNA internal transcribed spacer (ITS) region. Additional loci were amplified and sequenced based on their usefulness as phylogenetic markers for defining species in the DESC (Udayanga et al. 2014b), including DNA-lysase (*Apn2*; *Apn2fw2* and *Apn2rw2*; Udayanga et al. 2014b), translation elongation factor 1- α (*TEF1 α* ; EF1-728F and EF1-986R; Carbone and Kohn 1999), and beta-tubulin (*BenA*; *Bt-2a* and *Bt-2b*; Glass and Donaldson 1995). Partial 18s nuc rDNA (SSU) and 28s nuc (LSU) rDNA genes were amplified and sequenced following

the methods of Nguyen et al. (2014). Amplification of histone-3 (*HIS*) was attempted using methods described in Udayanga et al. (2014b).

DNA was amplified using a PCR master mix consisting of 0.5 μ L 2 μ M dNTPs, 0.04 μ L 20 μ M forward primer, 0.04 μ L 20 μ M reverse primer, 1 μ L 1 \times Titanium *Taq* buffer (Clontech, Mountain View, CA, U.S.A.), 0.1 μ L 0.5 \times Titanium *Taq* enzyme (Clontech), and 1 μ L of DNA template mixed in 7.32 μ L sterile Millipore water totaling 10 μ L per reaction. PCR products were verified by agarose gel electrophoresis and sequenced with Big Dye Terminator (Applied Biosystems, Foster City, CA, U.S.A.).

Sequence contigs were assembled and trimmed using Geneious R6 v6.1.8 (Biomatters, Ltd., Auckland, New Zealand). Sequences of each locus were aligned using MAFFT v7 (Kato et al. 2005) and the resulting alignments trimmed and manually checked using BioEdit v7.2.5 (Hall 1999). All new sequences were subsequently accessioned in GenBank (Table 3.1) and the alignments and phylogenetic trees in TreeBASE (study no. 18762; www.treebase.org/treebase/index.html).

Phylogenetic analyses of single and concatenated gene data sets were performed with Bayesian inference. The most suitable sequence evolution models for each gene (HKY+G for *APN2*, *BenA*, and *TEF1 α* ; HKY+I for ITS; HKY+I+G for combined *APN2*, *BenA*, and *TEF1 α* dataset) were determined based on the optimal Akaike information criterion scores in MrModeltest 2.2.6 (Nylander 2004). Bayesian analysis was performed with MrBayes 3.2 (Ronquist et al. 2012) with *Diaporthe citri* AR3405 as the outgroup. For each locus, three independent analyses using four Markov Chain Monte Carlo (MCMC) chains (one cold and three hot) were run simultaneously for 3 \times

10⁶ generations (standard deviation of split frequencies <0.01), sampling every 500 generations. The first 25% of trees were discarded as burn-in and the remaining trees were kept and combined into one consensus tree with 50% majority rule consensus. Consensus trees were imported into FigTree v1.4.2 (Rambaut 2014) and exported as SVG vector graphics for assembly in Adobe Illustrator (Adobe System, San Jose, CA, U.S.A.).

Secondary metabolites

A 2% MEA slant of *D. maritima* DAOM 628553 was macerated in sterile distilled deionized water (ddH₂O) and an aliquot was used to inoculate (5% v/v) 15 × 250 mL Erlenmeyer flasks containing 50 mL of 2% ME (Bacto, Becton, Dickinson and Company, Le Pont de Claix, France) broth. Flasks were incubated on a rotary shaker (100 RPM) for one week in the dark at 25 °C. Cultures were macerated, individually transferred to Glaxo bottles containing 1 L of the same liquid medium and incubated without agitation as described above for seven weeks.

The liquid culture was separated from the mycelia by vacuum filtration through a Whatman #4 filter (Whatman GE Healthcare Ltd., Buckinghamshire, U.K.), saturated with NaCl, extracted with ethyl acetate (EtOAc), and filtered through a Whatman #1 filter and anhydrous Na₂SO₄ prior to drying by rotary evaporation. The crude extract was dissolved in a minimal amount of HPLC grade methanol (MeOH), passed through a 13 mm PTFE syringe filter (Tisch Scientific, OH, U.S.A.) and dried under a gentle stream of

nitrogen gas yielding 1.6 g of crude extract. The dried extract and lyophilized mycelia were stored at $-20\text{ }^{\circ}\text{C}$ until needed.

An aliquot of crude extract was dissolved (5 mg mL^{-1}) in HPLC grade MeOH and analyzed by LC-UV-MS to access the secondary metabolite profile. This was achieved with a Waters 2795 separations module, Waters 996 diode array detector and Waters MicroMass Quattro LC mass spectrometer (Waters Corporation, Milford, MA, U.S.A.). Compounds were separated by a Kinetex C18 ($100\text{ x }4.6\text{ mm}$, $2.6\text{ }\mu\text{m}$) column (Phenomenex Inc., Torrance, CA, U.S.A.) with a mobile phase consisting of acetonitrile (ACN)-ddH₂O with formic acid (FA); (0.1% v/v). The linear solvent gradient was programmed from 5–100% ACN over 13 min with a flow of 1.0 mL min^{-1} .

The EtOAc culture filtrate extract (1.6 g) was fractionated by flash column chromatography utilizing a short silica gel column employing a step gradient elution with hexanes-EtOAc (0–100%; v/v) in 10% increments followed by 5, 10, 20, and 50% EtOAc-MeOH (v/v) yielding nine fractions. Fractions were subsequently screened by LC-UV-MS and three were selected for metabolite isolation by semi-preparative HPLC. Fraction 3 (131.3 mg) eluted with 20% EtOAc in hexanes (v/v) was further separated by semi-preparative HPLC with a linear gradient programmed from 15–100% ACN-ddH₂O over 15 min yielding phomopsolide A (25.0 mg; **1**) and compound **4** (5.8 mg). Fraction 4 (504.0 mg) eluted with 30% EtOAc in hexanes (v/v) and was separated by a similar HPLC method programmed from 30–60% ACN-ddH₂O over 16 min yielding phomopsolide C (4.6 mg; **3**). Fraction 5 (227.9 mg) eluted with 40% EtOAc in hexanes (v/v) and was

chromatographed with an HPLC method programmed from 25–100% ACN-ddH₂O over 18 min yielding phomopsolide B (15.3 mg; **2**).

Metabolite NMR spectra were obtained on a Bruker Avance 400 Spectrometer (Bruker Instruments Inc., Milton, ON, Canada) at 400.13 (¹H) and 100 MHz (¹³C) using a 5 mm auto-tuning broadband probe with a Z-gradient. Secondary metabolites were dissolved in CD₃OD (δ H 3.30 and δ C 49.0) (CDN Isotopes, Point Claire, QC, Canada) and were referenced to the solvent peak. Semi-preparative HPLC was performed with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a diode array detector and compounds were separated by a Luna C18 (250 × 10.0 mm, 5.0 μ m; Phenomenex Inc.) column with a mobile phase consisting of ACN-ddH₂O. Elution profiles were metabolite specific; however, each was linear programmed with a flow of 4.0 mL min⁻¹. LC-HRMS data was acquired with a Thermo Q-Exactive Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, U.S.A), coupled to an Agilent 1290 HPLC system. Metabolites were separated by an Agilent Zorbax Eclipse Plus RRHD C18 (2.1 × 50 mm, 1.8 μ m). Silica gel (Silicycle; 40–60 μ m) was utilized for metabolite separations. UV spectra of all major metabolites were obtained using a Varian Cary 3 UV-visible spectrophotometer scanning from 190–800 nm. Optical rotations were determined using an Autopol IV polarimeter (Rudolph Analytical, Hackettstown, NJ, U.S.A.).

Antimicrobial Assays

Initial antifungal activity of *Diaporthe maritima* DAOMC 628553 and DAOMC 250334 crude extracts (50 mg mL⁻¹) were screened using the Oxford diffusion assay

against *Microbotryum violaceum* and *Saccharomyces cerevisiae* homogeneously dispersed on 2% MEA (Vincent and Helen 1944). Subsequently, purified compounds **1-4** were tested for in vitro antimicrobial activity against *M. violaceum*, *S. cerevisiae*, *Bacillus subtilis* (ATCC 23857) and *Escherichia coli* (ATCC 67878). *Microbotryum violaceum* was grown in 20 g L⁻¹ malt extract (Bacto), 2.5 g L⁻¹ peptone (Bacto) and 2.5 g L⁻¹ yeast extract (Sigma, St. Louis, MO, U.S.A.) whereas *S. cerevisiae* was inoculated and grown in 1 g L⁻¹ yeast extract supplemented with 10 g L⁻¹ glucose. Bacteria were inoculated and grown in 5 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, and 10 g L⁻¹ NaCl. Nystatin was the positive control for antifungal assays and chloramphenicol was the antibiotic positive control. DMSO was the negative control for all assays. Metabolites were individually tested at 250, 25, and 2.5 µM in sterile 96-well microplates (Falcon 353072 Microtest-9, Franklin Lakes, NJ, U.S.A.). A 10 µL aliquot of each individual metabolite solution dissolved in DMSO was added to 200 µL fungal suspension. Assays were performed in triplicate and incubated at 28 °C with a rotary table shaker providing gentle agitation (500 rpm). Optical density (OD) measurements were made at 600 nm with a Molecular Devices Spectra Max 340PC reader (Sunnyvale, CA, U.S.A.). Antimicrobial OD data were analyzed by ANOVA followed by Tukey's test ($p < 0.05$) for significant differences (Systat V13.1; Systat Software Inc., Chicago, IL, U.S.A.) compared to the negative control (DMSO).

RESULTS

The *Diaporthe* species of interest was represented by 134 cultures isolated from surface-sterilized *P. mariana* and *P. rubens* needles and four cultures isolates from pycnidia erumpent on dead tips of otherwise healthy *P. rubens* branches (Fig. 3.1).

Pycnidia formed in culture on all media but perithecia were never observed. *Diaporthe* pycnidia were erumpent on the twig surface or petioles of dying or dead *Picea rubens* branch tips in the field or in branches incubated in moist chambers. Cultures derived from these specimens were conspecific with the endophyte isolates based on morphology and *Apn2*, *TEF1 α* , ITS, and *BenA* sequence similarity. Based on ITS BLAST searches in GenBank, this species was closely related to *D. celastrina* (identities=573/576 i.e. 99 %, gaps = 2/576) and other members of the DESC.

The *Apn2*, *TEF1 α* , ITS, and *BenA* sequence alignments consisted of 768, 359, 513, and 430 characters, respectively, from 23 isolates representing 12 *Diaporthe* species. HIS failed to amplify despite repeated attempts and PCR troubleshooting. All gene trees confirmed that our *Diaporthe* species was part of the DESC and closely related to *D. bicincta* and *D. celastrina* (Figs. 3.2–3.6), e.g.: *Apn2* *D. bicincta* (identities =766/768 i.e. 99 %, gaps = 0/768) and *D. celastrina* (identities =760/768 i.e. 99 %, gaps = 0/768); *BenA* *D. bicincta* identities =429/430 i.e. 99 %, gaps = 0/430) and *D. celastrina* (identities 429/430 i.e. 99 %, gaps = 0/430); and *TEF1 α* *D. bicincta* (identities =329/334 i.e. 99 %, gaps = 3/334) and *D. celastrina* (identities =328/335 i.e. 99 %, gaps = 4/335), although there was discrepancy in the placement of *D. celastrina* in the *Apn2* phylogeny (Fig. 3.3). The concatenated gene tree (*Apn2*, *TEF1 α* , *BenA*) strongly supported both the relationship between *D. maritima*, *D. bicincta*, and *D. celastrina* and the distinctiveness of *D. maritima* (Fig. 3.6). Some discordance was observed among the individual gene trees, for example the *BenA* phylogeny showed a polytomous clade consisting of *D.*

alleghaniensis, *D. eres*, *D. helicis*, *D. pulla*, and *D. vaccinii* (Fig. 3.4), species which generally formed distinct clades in the other gene trees.

From the culture filtrate extract of *D. maritima* DAOMC 628553, an endophyte isolated from healthy *P. rubens* needles, three dihydropyrone, phomopsolide A (**1**), B (**2**) and C (**3**), and a stable alpha pyrone (**4**) were characterized (Fig. 3.7). Based on spectroscopic data, several other related natural products were also produced; however, these metabolites were chemically unstable. The structures of compounds **1–4** were elucidated by interpretation of NMR, UV, and OR spectroscopic data mass spectrometry, and comparisons with the literature. The chemical profile of *D. maritima* DAOMC 250034, another strain isolated from healthy *P. rubens* needles as part of our investigation of foliar endophytes, produced the same natural products as *D. maritima* DAOMC 628553. Using mellein and tyrosol isolated from CBS 120379 (*Rhytismataceae* sp.) as standards, neither metabolite could be detected by HRMS from the *Diaporthe* extracts studied here.

The crude extracts of *D. maritima* DAOMC 628553 and DAOMC 250334 both inhibited the growth of the biotrophic pathogen *M. violaceum* and the yeast *S. cerevisiae*. Compounds **1–4** were tested individually for in vitro antimicrobial activity to determine if they significantly ($p < 0.05$) reduced the growth of *M. violaceum*, *S. cerevisiae*, *B. subtilis* (ATCC 23857), and *E. coli* (ATCC 67878). Phomopsolide A (**1**) was active against both *M. violaceum* and *S. cerevisiae* at 25 μ M whereas phomopsolide B (**2**), C (**3**), and **4** demonstrated growth inhibition at 250 μ M. Compound **1** showed antibiotic activity towards *B. subtilis* at 2.5 μ M whereas compounds **2–4** were active at

25 μM . None of the isolated metabolites inhibited the growth of the Gram-negative bacterium *E. coli* at the concentrations tested.

Physical data for compounds 1–4

Phomopsolide A (**1**): 25.0 mg; light yellow oil; $[\alpha]_D^{25}$ 366 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (4.41), 320 (3.12); ^1H and ^{13}C NMR spectroscopic data in accordance with Grove (1985), Stierle et al. (1997); HRMS m/z 295.11758 $[\text{M}+\text{H}]^+$ (calc. for $[\text{C}_{15}\text{H}_{19}\text{O}_6]^+$ 295.1176).

Phomopsolide B (**2**): 15.3 mg; light yellow oil; $[\alpha]_D^{25}$ 136 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 224 (3.62), 326 (2.10); ^1H and ^{13}C NMR spectroscopic data in accordance with Grove (1985), Stierle et al. (1997); HRMS m/z 297.13327 $[\text{M}+\text{H}]^+$ (calc. for $[\text{C}_{15}\text{H}_{121}\text{O}_6]^+$ 297.1333).

Phomopsolide C (**3**): 4.6 mg; light yellow oil; $[\alpha]_D^{25}$ 63 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (3.53), 320 (2.68); ^1H and ^{13}C NMR spectroscopic data in accordance with Stierle et al. (1997); HRMS m/z 295.11758 $[\text{M}+\text{H}]^+$ (calc. for $[\text{C}_{15}\text{H}_{19}\text{O}_6]^+$ 295.1176).

(*S,E*)-6-(4-hydroxy-3-oxopent-1-en-1-yl)-2H-pyran-2-one (**4**): 12.2 mg; clear oil; $[\alpha]_D^{25}$ -2 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 240 (3.64), 330 (3.75); ^1H and ^{13}C NMR spectroscopic data in accordance with Grove (1985); HRMS m/z 195.06534 $[\text{M}+\text{H}]^+$ (calc. for $[\text{C}_{10}\text{H}_{11}\text{O}_4]^+$ 195.0652).

TAXONOMY

Diaporthe maritima J.B. Tanney, sp. nov. Fig. 3.1.

MycoBank: MB 816941

Typification. Canada, New Brunswick, Alma, Fundy National Park, Dickson's Falls, 45.58690°N, 64.97468°W, 100 m alt, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 23 Sept 2013, *J.B. Tanney NB-365-711* (holotype DAOM 695742; isotypes DAOM 695742, DAOM 695743). Ex-type culture DAOMC 250563.

Etymology: From Latin *maritima*, maritime, referring to the frequent isolation and collection of this fungus on the Bay of Fundy coast in New Brunswick, Canada.

Colonies on MEA after 7 d at 25 °C reaching 5 cm diam, flat, margin undulate, aerial mycelia sparse, surface and reverse white to pale grey with greyish yellow (1B5) centre, exudates absent. Development of pycnidia in culture highly variable among strains; in fertile strains, pycnidia are produced within 2–6 wk, frequently sterile, scattered, columnar, up to 5 mm tall.

Conidiomata pycnidial, globose to subglobose, eustromatic, unilocular to convoluted or multilocular, immersed to semi-immersed, scattered or aggregated, dark brown to black, ostiolate, up to 300 µm diam, outer surface sometimes covered with hyaline hyphae; pycnidial wall 60–140 µm wide, consisting of (3–)5–9(–14) µm diam, light to dark brown, thick-walled, pseudoparenchymatous cells, outer layers thick-walled and heavily pigmented, inner layers becoming hyaline and more compressed toward pycnidial cavity; conidial mass exuding in yellowish white to pastel yellow

(3A2–3A4) cirrhi from ostioles. Conidiogenous cells phialidic, subcylindrical to ampulliform, straight to sinuous, cylindrical or slightly tapering towards the apex, collarette flared, periclinal thickening observed, (8.5–)9–12.5(–16) × 2–3 μm (n = 50). Paraphyses not observed. Alpha conidia aseptate, hyaline, smooth, oblong to fusiform or ellipsoidal, apex bluntly rounded, base subtruncate, bi- to multi-guttulate, (10–)11–12.5(–13.5) × (3–)3.5–4 μm (n = 50). Beta conidia aseptate, hyaline, smooth, straight to hamiform or uncinata, (29–)32–38(–40) × (1–)1.5–2 μm (n = 50). Gamma conidia not observed.

Cardinal temperatures: Range 5–35 °C, optimum 20 °C, minimum <5 °C, maximum slightly >35 °C.

Host range: Endophyte of healthy *Picea mariana* and *P. rubens* needles and associated with dying or recently dead twigs of *Picea mariana* and *P. rubens*.

Distribution: Canada (New Brunswick).

Additional specimens and cultures examined: See Supplementary Table 3.1.

Notes: *Diaporthe maritima* was isolated as an endophyte from surface-sterilized needles and from pycnidia collected in the field. Pycnidia were commonly associated with dead or dying twigs and found erupting through twig surfaces and petiole apices where needle abscission occurred. It is unknown whether *D. maritima* is a pathogen causing twig death, an opportunistic pathogen, or a saprotroph. *Diaporthe maritima* belongs to the *D. eres* species complex and differs morphologically from the related species *D. bicincta* and *D. celastrina* by the production of beta conidia and by the dimensions of conidiophores and alpha conidia.

DISCUSSION

Several *Diaporthe* species are associated with twigs, cones, and foliage of *Pinaceae* species, including *D. conigena*, *D. conorum*, *D. griseotringens*, *D. larseniana*, *D. lokoyae*, *D. nobilis*, *D. occulta*, *D. pinicola*, *D. pinophylla*, *D. pithya*, and *D. thujana* (Wehmeyer 1933, Hahn 1943, Farr and Rossman 2016). Of these, *D. conorum*, *D. larseniana*, *D. occulta*, and *D. pithya* are reported from *Picea*; however, accessioned sequences were only found for purported *D. conorum* (e.g.: AB201443, DQ116551, DQ116552, FJ158120, JQ814306) and *D. occulta* (e.g.: HM439635) strains. None of these accessioned ITS sequences were conspecific with *D. maritima*, e.g.: *Diaporthe conorum* CBS 186.37, isolated from *Picea abies* seedlings in England, has a 98% similarity (identities=500/509, gaps=3/509). Comparison of sequences should preferably be based on ex-types or epitypes; however, apparently no such validated cultures exist for these *Picea*-associated species. Ko et al. (2011) mentioned a purported ex-epitype of *D. conorum*; however, this species is not closely related to the DESC. Efforts to generate epitypes and ex-epitype strains for *Diaporthe* species are currently underway (e.g.: Udayanga et al. 2014a, Udayanga et al. 2014b, 2015). Epitypification of poorly characterized conifer-associated *Diaporthe* species using specimens derived from the original host and type locale will help resolve species identification and classification.

Diaporthe maritima differs morphologically from other reported species on *Picea* by its production of larger conidia compared to *D. conorum* (alpha conidia 6.5–13 × 2.5–4 µm; beta 10–32 × 1 µm) (Hahn 1928) and *D. occulta* (6–8 × 2–2.5 µm; beta 15–34.5 µm) (Hahn 1930). Hahn (1928) described the beta conidia of *D. conorum* as

horseshoe-shaped, while those of *D. maritima* are straight to hamiform or uncinata. The closely related species *Diaporthe bicincta* and *D. celastrina* both produce alpha conidia of similar size (9–12 × 2–3.5 μm) to *D. maritima*, but beta conidia have not been observed in either species. The distinctiveness of *D. maritima* is supported by morphological characters and the phylogenetic analyses using *Apn2* and *TEF1α* loci, which were found to show the highest degree of informativeness per site by Udayanga et al. (2014b)

Species within *Diaporthe* represent multi-host endophytes and/or tree-associated fungi that may be observed on unrelated trees from different geographical locations (Webber and Gibbs 1984, Rehner and Uecker 1994, Pirttilä and Frank 2011). The production of biologically active secondary metabolites by this genus no doubt factors into this prominent plant-endophyte interaction. From *D. maritima* DAOMC 628553, isolated as a needle endophyte of *P. rubens*, phomopsolides A (**1**), B (**2**), C (**3**) and a stable alpha-pyrone (**4**) were structurally characterized. Phomopsolides A (**1**) and B (**2**) were originally isolated from *Diaporthe eres* (as *Phomopsis oblonga*), a fungal species commonly associated with the outer bark of healthy *Ulmus* trees (Grove 1985). DESC strains have been shown to invade the phloem tissue of *Ulmus* trees infected with the fungus *Ophiostoma ulmi*, the causative agent of Dutch elm disease (Webber 1981). Phomopsolides A (**1**), B (**2**) and compound **4** were demonstrated to elicit anti-feedant and anti-boring activity against adult female bark beetles (*Scolytus scolytus*), an insect vector of *O. ulmi* (Claydon et al. 1985). As compound **4** retained activity, it indicated the tyglic acid moiety is not required for antiinsectan activity. These fungi were

hypothesized to naturally control Dutch elm disease when *Diaporthe* species disrupted the breeding of the bark beetle, the vectors of the fungal pathogen (Webber 1981, Webber and Gibbs 1984).

Subsequent to the original discovery of phomopsolides from species within the DESC, this class of secondary metabolites has been reported from related and phylogenetically distant fungi associated with various host species. Phomopsolide C (**3**), the *trans* isomer of **1**, was originally isolated from a *Penicillium* sp. (NRRL 21208) obtained from the inner bark of the Pacific yew (*Taxus brevifolia*). Phomopsolides A–C (**1–3**), phomopsolides D–E and a furanone were also characterized from this strain. All isolated metabolites had strong inhibitory activity towards *Staphylococcus aureus*, comparable to the known antibiotics tetracycline and streptomycin (Stierle et al. 1997). All phomopsolides isolated from *D. maritima* had strong antimicrobial activity towards a Gram-positive bacterium, *B. subtilis*, where phomopsolide A (**1**) was the most potent. Phomopsolide B (**2**) was recently characterized as the dominant metabolite from an undescribed *Diaporthe* species (GenBank DQ272498) isolated from surface-sterilized twig tissue of *Camptotheca acuminata* (Yuan et al. 2009). Nine *Diaporthe* strains associated with grape vines growing in Switzerland synthesized several different mixtures of secondary metabolites. Characterized natural products included phomopsolide B, four furanones, the xanthenes sydowinin A and sydowinol, and cytosporone (Goddard et al. 2014). A large proportion of North American and European *Diaporthe* endophytes of *Salix* trees produced phomopsolide B whereas phomodiol synthesis varied (Horn et al. 1996). Because of their biological activities and intriguing

structures, this class of compound has been the target of multiple chemical syntheses (Harris and O'Doherty 2002, Li et al. 2004, Michaelis and Blechert 2005, Prasad and Gutala 2012).

Diaporthe species associated with woody plants produce a diverse array of biologically active natural products. Several of these chemicals are directly implicated in host tree-pest chemical ecologies. From these chemical investigations, phomopsolide B appears as the most commonly reported metabolite from *Diaporthe* species (Claydon et al. 1985, Grove 1985, Horn et al. 1996). The secondary metabolites of European isolates within the DESC associated with *Fraxinus*, *Platanus*, and *Ulmus* trees were studied because of the known antiinsectan activity of their natural products including phomopsolides (Claydon et al. 1985, Grove 1985). Chemical differences were observed between morphologically distinct *Ulmus*-derived isolates. *Diaporthe* isolates from each of the host trees produced phomopsolide B (**2**) and oblongolide (Begley and Grove 1985, Claydon et al. 1985, Grove 1985). The *Ulmus*-derived *Diaporthe* species additionally synthesized various pyrones, orsellinic acids, aromatics, and isocoumarins including (*R*)-5-methylmellein, tyrosol, and mellein. Interestingly, several of these secondary metabolites were also reported from endophytes of *Picea glauca* in the Acadian forest of North America (Sumarah et al. 2008b). Culture filtrate extracts of these *P. glauca* endophytes, when incorporated into the diets of *Choristoneura fumiferana* (Eastern spruce budworm) larvae, significantly reduced both weight and head capsule size (Sumarah et al. 2008b). Phomopsolides A (**1**) and B (**2**), (*R*)-5-methylmellein, tyrosol, and mellein all displayed in vitro antiinsectan activities, where phomopsolide A was the most

active, towards the elm bark beetle *S. scolytus* (Claydon et al. 1985). Of particular interest is the identification of the same biologically active natural products synthesized by different tree-associated fungi of different geographic origin having potentially very important ecological roles. These data further demonstrate the production of antagonistic mixtures of secondary metabolites by tree-associated and endophytic fungi toward host insect pests.

Diaporthe maritima was commonly found on dying or dead branch tips in the sampling sites and has probably been encountered by other researchers in North America. However, because of its position within the DESC, it is conceivable that its novelty was overlooked. Endophytes of forest understory plants within the study sites have not been sampled; therefore host specificity or host range of *D. maritima* is undetermined. Furthermore, the interactions between *D. maritima* and its plant host are currently unknown; however, subsequent field inoculation studies are planned to assess production of phomopsolides *in planta* and the effects of *D. maritima* needle infection on budworm damage and host health. The production of potent antiinsectan secondary metabolites by foliar endophytes such as *D. maritima* may provide integrated pest management tools that support economically and environmentally sustainable forestry practices in Canada.

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AUTHOR CONTRIBUTIONS

JBT and DM conceived and designed the work. JBT acquired the mycological data, provided strains, collections, and sequences from Canadian material; JBT provided the species descriptions, tested all markers and generated all sequences, conducted cultural studies and phylogenetic analyses, deposited cultures in DAOMC and specimens in DAOM. DM, BG, and JDM were responsible for the large scale fermentation of the *Diaporthe* strains, the isolation and characterization of phomopsolides A-C and the stable alpha pyrone, and discussion of these biologically active secondary metabolites. JBT and KAS interpreted taxonomic considerations. JBT and DVM drafted the article; all authors critically revised the manuscript for content.

Chapter 4: Phacidiaceae endophytes of *Picea rubens* in Eastern Canada

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ABSTRACT

More than 100 endophyte strains belonging to the family Phacidiaceae were isolated from surface-sterilized *Picea rubens* needles from the Acadian Forest Region in New Brunswick, Canada. Strains were characterized morphologically by their asexual states and phylogenetic analyses were conducted using the nuc internal transcribed spacer rDNA (ITS) barcode and the second largest subunit of ribosomal polymerase II (*RPB2*). Morphological and phylogenetic data revealed seven species: *Darkera* cf. *parca*, *Strasseria geniculata*, two novel *Phacidium* species, and three novel monotypic genera described to accommodate distinct species: *Calvophomopsis rubensicola*, *Cornibusella ungulata*, and *Gloeopycnis protuberans*. Further analyses with *Darkera* spp. were performed with ITS and partial translation elongation factor 1- α (*TEF1 α*), suggesting *Darkera parca* is a species complex. Phacidiaceae is a large family comprising hundreds of species that are unrepresented by sequence data; therefore, ITS barcodes were generated from herbarium material including type specimens of *Darkera parca* and *Phacidium lunatus* and specimens of other Phacidiaceae species in *Allantophomopsiella*, *Allantophomopsis*, *Bulgaria*, *Phacidium*, and *Pseudophacidium*. The description of novel species combined with morphological observations and reference sequence barcodes will facilitate the identification of conifer endophytes and improve our understanding of the large and mostly neglected family Phacidiaceae.

Keywords

coelomycete; *Ceuthospora*; *Phacidium*; molecular taxonomy; species identification;
systematics

INTRODUCTION

Phacidiaceae (Phacidiales, Leotiomycetes) was previously relegated to a taxonomic dumping ground comprised of phylogenetically heterogeneous taxa that share superficial similarities, namely immersed ascomata that open to reveal the hymenia by splitting of the covering stromatic layers or superficial host tissues (Korf 1962, Sherwood 1977, Dicosmo et al. 1983). For example, the distantly related genera *Therrya* (Rhytismataceae, Rhytismatales) and *Rhabdocline* (Cenangiaceae, Helotiales) were once considered members of the Phacidiaceae based on morphological similarities (Dennis 1968, Funk 1980a). Barr (1976) noted that the Phacidiaceae was one of the most difficult groups of genera to arrange, tentatively placing the family in the Phyllachorales. Seminal work by Dicosmo et al. (1983, 1984) resulted in a more clearly defined family based on both ascomata and asexual state characters, which ultimately rejected hysteriaceous, rhytismataceous, and stictidiaceous genera that previously obscured a more cohesive and monophyletic concept and restricted the Phacidiaceae to *Lophophacidium* and *Phacidium*. Following Dicosmo's treatment, taxonomic work in the Phacidiaceae idled, resulting in a paucity of reference DNA sequences and sampling. However, recent phylogenetic studies have resolved some important problems pertaining to the taxonomy and nomenclature of the Phacidiaceae, including resurrecting the monotypic order Phacidiales, reducing the coelomycete genus *Ceuthospora* to synonymy with *Phacidium* in accordance with the single name nomenclature system, and the epitypification of the type species of *Allantophomopsis*,

A. cytispora (Hawksworth et al. 2011, Crous et al. 2014b, Crous et al. 2015a, Crous et al. 2015b).

The Phacidiaceae contains hundreds of described species, including >270 names in *Phacidium* and 125 names in *Ceuthospora*. Members of the Phacidiaceae are reported from a diverse group of plant hosts, particularly species within the Ericaceae, Rosaceae, and Pinaceae (Dicosmo et al. 1984, Funk 1985, Farr and Rossman 2016). Phacidiaceae species such as *Allantophomopsis cytispora*, *A. lycopodina*, *Phacidiopycnis washingtonensis*, and *Phacidium lacerum* are associated with disease and postharvest fruit rot of *Malus*, *Pyrus*, and *Vaccinium* (Carris 1990, Putnam 2005, Xiao et al. 2005, Vilka et al. 2009, Weber 2011, Wiseman et al. 2015). Species are also reported as endophytes, saprotrophs, and pathogens from conifer trees (Smerlis 1969, Whitney et al. 1975, Funk 1985, Karadžić 1998, Sokolski et al. 2007, Koukol et al. 2012). Overall, relatively little is known about the taxonomy and biology of the conifer-associated Phacidiaceae species.

Only ca. 20 Phacidiales spp. are represented by named sequences in GenBank: *Allantophomopsiella pseudotsugae*, *Allantophomopsis cytispora*, *A. lunata*, *A. lycopodina*, *Bulgaria inquinans*, *Ceuthospora pinastri*, *Darkera parca*, *D. piceae*, *Phacidium calderae*, *P. fennicum*, *P. grevilleae*, *P. lacerum*, *P. lauri*, *P. mollerianum*, *P. pseudophacidioides*, *P. trichophori*, *P. vaccinnii*, *Potebniamyces pyri*, and *Strasseria geniculata*. Numerous endophyte sequences accessioned in GenBank represent unidentified Phacidiaceae species from various studies (Stefani and Bérubé 2006, U'Ren

et al. 2010), suggesting a more prominent role of endophytism in the life history of Phacidiaceae species than previously recognized. Foliar endophytes associated with woody plants are notoriously difficult to identify, because morphological characters cannot be relied upon since most strains are sterile on standard media and sequence-based identification is problematic because of a lack of authentic reference sequences.

Saprotrophic and opportunistic pathogenic fungi may asymptotically infect host tissue until optimal conditions for extensive colonization or reproduction arise. Consequently, studies surveying endophytes from asymptomatic foliage may detect species that are unidentifiable based on culture morphology or DNA sequences, but actually represent endophytic life stages of named-but-unsequenced species. Accurate identification of endophytes that are unknown based on current reference sequence data is possible by connecting unknown endophyte sequences with identifiable field or herbarium specimens or by the induction of taxonomically informative morphological characters (Knapp et al. 2015, Tanney et al. 2016a).

In this study, 110 Phacidiaceae endophytes were isolated in pure culture from surface-sterilized healthy *Picea rubens* needles and characterized morphologically and phylogenetically. Phylogenetic analyses using the nuc internal transcribed spacer rDNA (ITS) barcode and the second largest subunit of ribosomal polymerase II (RPB2) combined with morphological characters of asexual states induced in vitro revealed seven species: *Darkera* cf. *parca*, *Strasseria geniculata*, two novel *Phacidium* species, and three novel monotypic genera to accommodate distinct species: *Calvophomopsis*

rubensicola, *Cornibusella ungulata*, and *Gloeopycnis protuberans*. ITS barcodes were generated from type specimens of *Darkera parca* and *Phacidium lunatus* and herbarium specimens of unrepresented species including *Ceuthospora phacidioides*, *Pseudophacidium ledi* originating from an ericaceous host, and *P. piceae*.

MATERIALS AND METHODS

Cultures

Endophytes were isolated from healthy, asymptomatic *Picea rubens* needles using the methods outlined by Tanney et al. (2016a). Cultures were maintained on 2% malt extract agar (MEA) at 16 °C and attempts to induce sporulation in vitro involved prolonged incubation on MEA, corn meal agar (CMA; Acumedia Manufacturers Inc., Lansing, MI), oatmeal agar (OA; Crous et al. 2009), spruce needle potato agar (SNPA; modified from Su et al. 2012), 1.5% water agar (WA, with 1 mL trace metal solution; Crous et al. 2009) with or without the addition of sterile *P. rubens* needles on the agar surface, 20% apple juice agar (AJA; modified from Liu and Xiao 2004), autoclaved *Picea rubens* twigs in 250 mL flasks containing moist perlite, and floating agar plugs containing mycelia in sterile distilled water under ambient light conditions. Cultures were incubated under several light regimes including 24 h dark, 12 h fluorescent light cycle, and ambient light. Representative strains were deposited in the Canadian Collection of Fungal Cultures (DAOMC).

Morphology

Vertical sections of fresh pycnidia were cut by hand or in 8–12 µm sections using a freezing microtome and mounted in either water, 85% lactic acid, 5% KOH, lactofuchsin, or cresyl blue. Microscopic measurements were taken from material mounted in water and are presented as ranges calculated from the mean ± standard deviation of each measured value, with outliers in brackets. Colony characters and diameters were recorded for 2-wk old cultures inoculated on MEA and incubated at 20 °C. Colony colors were described using the alphanumeric codes of Kornerup and Wanscher (1978). Observations were made using an Olympus BX50 light microscope and micrographs were captured using an Evolution MP Color Camera (Media Cybernetics, Silver Spring, CA, U.S.A.) and Image-Pro Plus v6.0 (Media Cybernetics) or InfinityX-32 camera (Lumenera Corp., Ottawa, ON, Canada) and Infinity Analyze v6.5.2 (Lumenera Corp.) software. Colony macrophotographs were captured with a Nikon Coolpix P5000 (Nikon Inc., Tokyo, Japan) and photographic plates were assembled using Adobe Photoshop v5.5 (Adobe Systems, San Jose, CA, U.S.A.).

DNA isolation, amplification and analyses

Total genomic DNA was extracted from 4–12-wk old cultures using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The primers ITS4 and ITS5 (White et al. 1990) were used to amplify and sequence the ITS region, fRPB2-5f2 and fRPB2-7CR to amplify and sequence *RPB2* (Liu et

al. 1999), and EF1-728F and EF1-986R to amplify and sequence partial translation elongation factor 1- α (*TEF1 α*) (Carbone and Kohn 1999). Amplification of *RPB2* using the primer pair fRPB2-5F and fRPB2-414R, as outlined by Crous et al. (2014b), was repeatedly attempted. Partial 28S nuc rDNA (LSU) gene (LSU) was amplified using the primer pairs LR0R and LR8 and sequenced using LR0R, LR3R, LR5, and LR7 (Vilgalys and Hester 1990). ITS was selected because it is the official primary barcoding marker for fungi and *RPB2* was chosen because it is capable of distinguishing closely-related species and is one of the few protein-coding genes represented by Phacidiales species in NCBI GenBank (Schoch et al. 2012, Crous et al. 2014b). LSU was sequenced because abundant reference sequences exist for this region, it can provide good generic or higher level taxonomic classification, and it may be aligned across distantly related taxa (Liu et al. 2012, Porter and Golding 2012). *TEF1 α* was sequenced for *Darkera* to examine interspecific and intraspecific variation because it is a promising candidate as a universal secondary barcode and *TEF1 α* sequences for *Darkera* species are available in GenBank (Crous et al. 2015b, Stielow et al. 2015). DNA was amplified using a PCR master mix consisting of 0.5 μ L 2 mM dNTPs, 0.04 μ L 20 μ M forward primer, 0.04 μ L 20 μ M reverse primer, 1 μ L 10 \times Titanium *Taq* buffer (Clontech, Mountain View, CA, U.S.A.), 0.1 μ L 50 \times Titanium *Taq* enzyme (Clontech), 1 μ L of DNA template, and 7.32 μ L sterile Milli-Q water (Millipore, Bedford, MA, U.S.A.) per reaction. All loci were amplified using the following PCR profile: 95 $^{\circ}$ C for 3 min, then 35 cycles at 95 $^{\circ}$ C for 1 min, 56 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 1.5 min, followed by a final extension at 72 $^{\circ}$ C for 10 min. PCR products

were verified by agarose gel electrophoresis and sequenced with Big Dye Terminator (Applied Biosystems, Foster City, CA, U.S.A.).

Whole or partial apothecia or 2 mm discs of symptomatic plant tissue were removed from dried herbarium specimens (Supplementary Table 4.1) and ground in liquid nitrogen using Axygen polypropylene pestles (PES-15-B-SI, Union City, CA, U.S.A.). Total genomic DNA was extracted using the manufacturer's recommended kit for a Thermo Scientific KingFisher mL magnetic particle processor (VWR, Mississauga, ON, Canada). The primers ITS4A and ITS5 (White et al. 1990, Larena et al. 1999) were used to amplify and sequence the ITS region. The previously described PCR profile and PCR master mix were used for amplification, with the exception of the addition of 0.5 μ M 20 mg/ml bovine serum albumin (BSA) per reaction.

Sequence contigs were assembled, trimmed, and manually checked using Geneious R6 6.1.8 (Biomatters, Auckland, New Zealand) and each individual gene dataset aligned using MAFFT v7 (Katoh et al. 2005). The resulting alignments were trimmed and manually checked using BioEdit v7.2.5 (Hall 1999).

Bayesian analyses were performed for each individual gene dataset using MrBayes v3.2 (Ronquist and Huelsenbeck 2003) and the most suitable sequence evolution models selected for each gene based on the optimal Akaike information criterion scores in MrModeltest v2.2.6 (Nylander 2004): SYM+I+G for ITS and *RPB2* and K80 for *TEF1 α* . For each locus, three independent Markov Chain Monte Carlo (MCMC) samplings were performed with four chains (three heated and one cold) with sampling

every 500 generations until the standard deviation of split frequencies reached a value <0.01. The first 25% of trees were discarded as burn-in and the remaining trees were kept and combined into one consensus tree with 50% majority rule consensus. Convergence was assessed from the three independent runs using AWTY and Tracer v1.6 (Wilgenbusch et al. 2004, Rambaut et al. 2014). Consensus trees were imported into FigTree (Rambaut 2014) and exported as SVG vector graphics for assembly in Adobe Illustrator v10 (Adobe System, San Jose, CA, U.S.A.).

RESULTS

Surface-sterilized healthy *Picea rubens* needles yielded 110 Phacidiaceae endophyte strains. Strains were initially grouped according to culture morphology and representatives selected for ITS sequencing and morphological characterization of asexual morphs. ITS sequences indicated at least five distinct species and *RPB2* sequences and morphological characterization confirmed the presence of *Strasseria geniculata*, two *Phacidium* spp., two *Phacidiopycnis*-like species, and one sterile species that produced distinct stromatic protuberances up to 1 cm tall. The two *Phacidium* spp. and three novel monotypic genera are described in the Taxonomy section below.

The ITS alignment consisted of 140 taxa and was 491 bp long, with the only significant indel region being a 20 bp deletion at position 90 for the *Darkera* strains. The overall topology of the ITS phylogenetic tree was polytomous and weak or moderately supported (Fig. 4.1). The *Phacidium* strains clustered together with little distinction between a number of species including *Phacidium dicosmosum*, *P. faxum*, *P. fennicum*,

P. lacerum, *P. lauri*, and *P. pseudophacidioides*. *Bulgaria* strains formed a strongly supported [posterior probability (P.P.) = 1] clade sister to *Phacidium* with a long branch length. *Allantophomopsiella pseudotsugae*, *Phaciopycnis washingtonensis*, and *Potebniamyces pyri* formed a strongly supported clade with *Allantophomopsis cytispora*, *A. lunata*, and *Allantophomopsis* sp. (CBS 322.36) occupying a weakly supported basal clade. The *Darkera* strains formed a well-supported long-branch clade and other species including *Allantophomopsis cytispora*, *A. lycopodina*, *Cornibusella ungulata*, *Pseudophacidium ledi*, *Ps. piceae*, and *Strasseria geniculata* formed polytomous clades within the Phacidiaceae. The *Clavophomopsis rubensicola* strains were very closely related (1 bp difference) to a *Phacidiopycnis* sp. strain (HM595538) isolated as an *Abies beshanzuensis* endophyte in China.

The *RPB2* alignment consisted of 41 taxa and was 689 bp long without intron regions. Based on the *RPB2* phylogeny, *Phacidium* spp. formed a polytomous clade with a strongly supported backbone and included *Bulgaria inquinans*, probably resulting from long branch attraction (Fig. 4.2). *Phacidium dicosmum*, *P. faxum*, and *P. fennicum* were closely related and formed a strongly supported clade (P.P. = 1). *Calvophomopsis rubensicola* and *Gloeopycnis protuberans* were sister to *Darkera* isolates although the support between these genera was only moderate (P.P. = 0.88). *Cornibusella ungulata* was basal to the Phacidiaceae species included in this analysis. Attempts to amplify partial *RPB2* using the reverse primer fRPB2-414R reported in Crous et al. (2014b) failed despite many PCR troubleshooting attempts. The entire *RPB2* dataset of Crous et al. (2014b) could not be used in this study because there was little overlap in alignments

containing *RPB2* sequences amplified using the standard *RPB2* primer pair in this study and those of Crous et al. (2014b). The sequences generated from fRPB2-5F and fRPB2-414R are only ca. 355 bp and have poor coverage with other *RPB2* sequences in GenBank; therefore, the fRPB2-414R reverse primer should be avoided and standard *RPB2* primers used instead.

The *TEF1α* alignment included 25 *Darkera* isolates and was 397 bp long with a small gap-filled region resulting from a 2 bp insertion at position 101 for the *Darkera parca* holotype (DAOM 145413) and an 11 bp deletion at position 92 for the New Brunswick *Darkera cf. parca* isolates. The phylogeny showed the distinction of the *Darkera parca* type specimen, purported *D. parca* Siberian isolates, and the New Brunswick *D. cf. parca* isolates from *Picea rubens* (Fig. 4.3). *Darkera picea* were more diverse but only differed from one another by a maximum of 3 bp, e.g.: CPC 23887 and CPC 23892; identities = 392/395 (99%), gaps = 0/395 (0%). The *Darkera parca* holotype and Siberian “*Darkera parca*” strains were the least similar, e.g.: CPC 23904; Identities = 362/370 (98%), gaps = 4/370 (1%).

TAXONOMY

Phacidium dicosmosum J.B. Tanney, sp. nov. Fig. 4.4.

MycoBank: MBXXXXXX

Typification. Canada: New Brunswick, Alma, Fundy National Park, Coppermine Trail, 45.5493 -65.01878, isolated as endophyte from healthy surface-sterilized *Picea*

rubens needle, 16 Jul 2014, J.B. Tanney RS9-5D (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC 250749.

Etymology: Named for Frank DiCosmo in honour of his seminal work on the Phacidiaceae.

Colonies 30–40 mm diam after 10 d in the dark at 20 °C on MEA; flat, very sparse sparse hyaline aerial mycelia, margin diffuse, flat, wide, entire to slightly sinuate, hyaline; surface and reverse dull yellow to olive brown (3B3–4E8) toward centre. Exudates and soluble pigments absent.

Conidiomata in MEA pycnidoid, scattered, dark brown (8F8) to black, up to 1400 µm diam; 4–6 locules (200–300 µm diam); wall up to 100 µm thick, dark brown (8F8), pseudoparenchymatous, gelatinized; cells of outer layer brown, thick-walled, textura intricata, textura globulosa, or textura angularis; cells of inner layer hyaline to subhyaline; no definite ostiole present, dehiscent by irregular tearing of the covering layer. Conidiophores arising from inner hyaline layers of locule, hyaline, smooth, simple or extensively branching sympodially, up to 40 µm long, 2–2.5 µm diam, invested in mucus. Conidiogenous cells discrete, cylindrical to subcylindrical, prominent periclinal thickening or percurrent proliferation, hyaline, smooth, (8.5–)10–14(–16.5) × (1.5–)2–2.5(–3) µm. Conidia aseptate, hyaline, smooth, thin-walled, oblong to oblong-ellipsoidal, tapering at ends, apex subobtuse, base subobtuse to subtruncate, (10–)11–12.5(–13.5) × (2–)2.5–3 µm, bearing a funnel-shaped mucoid apical appendage (2.5–)3–5(–6.5) µm

long, 2–4(–5.5) μm wide at the apex, often with 2–3 guttules 1.5–2 μm diam, exuded in yellowish white (3A2) droplets.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: see Supplementary Table 4.1.

Notes: *Phacidium dicosmosum* is most closely related to *P. faxum*, differing by its smaller conidia, (10–)11–12.5(–13.5) \times (2–)2.5–3 μm vs. (10.5–)12.5–15(–17) \times (2.5–)3–3.5(–4) μm (*P. faxum*), and more restricted radial growth. *RPB2* sequences easily distinguish both species: identities = 668/689 (97%), gaps = 0/689 (0%).

Phacidium faxum J.B. Tanney, sp. nov. Fig. 4.5

Mycobank: MBXXXXXX

Typification. Canada: New Brunswick, Alma, Fundy National Park, Coppermine Trail, 45.5493 -65.01878, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 16 Jul 2014, J.B. Tanney RS9-5Q (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC 250750.

Etymology: From Latin *faxum*, firebrand or torch, referring to the silhouette of the conidium and apical appendage.

Etymology: From Latin faxum, firebrand or torch, referring to the silhouette of the conidium and apical appendage.

Colonies 49–53 mm diam after 10 d in the dark at 20 °C on MEA; flat, very sparse hyaline aerial mycelia, margin diffuse, flat, wide, broadly sinuate, hyaline; surface and reverse olive brown to greyish brown (4D4–5D3) toward centre, sometimes predominately hyaline with olive brown to greyish brown (4D4–5D3) sectors, hyaline concentric rings sometimes observed. Exudates and soluble pigments absent.

Conidiomata in MEA pycnidoid, scattered, dark brown (8F8) to black, up to 1100 µm diam; 4–6 locules (130–300 µm diam); wall up to 100 µm thick, dark brown (8F8), pseudoparenchymatous, gelatinized; cells of outer layer brown, thick-walled, textura intricata, textura globulosa, or textura angularis; cells of inner layer hyaline to subhyaline; no definite ostiole present, dehiscent by irregular tearing of the covering layer. Conidiophores arising from inner hyaline layers of locule, hyaline, smooth, simple or extensively branching sympodially, branching angle acute, up to 50 µm long, 2–2.5 µm diam, invested in mucus. Conidiogenous cells discrete, cylindrical to cylindrical-ellipsoid, conspicuous periclinal thickening or percurrent proliferation, hyaline, smooth, 8–12(–15) × (1.5–)2–2.5(–4) µm. Conidia aseptate, hyaline, smooth, thin-walled, oblong to oblong-ellipsoidal, tapering at ends, apex subobtuse, base subobtuse to subtruncate, (10.5–)12.5–15(–17) × (2.5–)3–3.5(–4) µm, bearing a funnel-shaped mucoid apical appendage (2–)2.5–4(–5) µm long, (2.5–)3–4.5(–5) µm wide at the apex, often with 2–4(–5) guttules 2 µm diam, exuded in yellowish white to pale yellow (4A2–4A3) droplets.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: see Supplementary Table 4.1.

Notes: *Phacidium faxum* shares similar ITS sequences to ex-type sequences of *P.*

fennicum [CBS 457.83; identities = 522/523 (99%), gaps = 1/523 (0%), *P. grevilleae* [CBS 139892; identities = 581/583 (99%), gaps = 0/583 (0%)], *P. lacerum*, and *P.*

pseudophacidioides [CBS 590.69; identities = 520/522 (99%), gaps = 0/522 (0%)].

Phacidium fennicum was described from dead *Pinus sylvestris* needles in Finland and has longer conidia than *P. faxum* (16–18 × 3 µm) (Butin and Soderholm 1984). Compared to *P. grevilleae*, a species described from *Grevillea robusta* leaves in Uganda, *Phacidium faxum* has longer conidiogenous cells, 8–12(–15) × (1.5–)2–2.5 µm vs. 5–8 × 2–3 µm (*P. grevilleae*), and shorter conidia, (10.5–)12.5–15(–17) × (2.5–)3–3.5(–4) µm vs. (15–)17–19(–22) × (2.5–)3 µm (*P. grevilleae*), (Crous et al. 2015d). The type species of *Phacidium*, *P. lacerum*, was epitypified by Crous et al. (2014b) with a specimen from *Pinus sylvestris* needles in France. The conidiogenous cell and conidia dimensions of *P. lacerum* provided by Crous et al. (2014b) are remarkably similar to those of *P. faxum*, however the *RPB2* sequences vary significantly [CBS 761.73; identities = 647/689 (94%); gaps = 0/689 (0%)]. *Phacidium faxum* differs from *P. pseudophacidioides*, described from *Ilex aquifolium* in the Netherlands, by its large conidia with more variable

dimensions, (10.5–)12.5–15(–17) × (2.5–)3–3.5(–4) μm vs. (11–)11.5–12.5(–13) × (2–)2.5 μm (*P. pseudophacidioides*). *Phacidium faxum* shares similar ITS sequences to specimens identified as *Phacidium mollerianum* [e.g.: CBS 574.66; identities = 520/522 (99%), gaps = 0/522 (0%)]; *P. mollerianum* was originally described from *Eucalyptus* sp. leaves in Italy and has smaller conidia, (9–)10–12(–13) × (2–)2.5 μm (Crous et al. 2014b).

Calvophomopsis J.B. Tanney, gen. nov.

Mycobank: MBXXXXXX

Etymology: Latin for bald, denoting the lack of conidial appendages typical for many Phacidiaceae genera.

Diagnosis: Distinguished from *Allantophomopsis* and *Allantophomopsiella* by the flaring phialide collarettes and ellipsoidal conidia lacking appendages, from *Phacidiopycnis* by the absence of microcyclic conidiogenesis, conidia budding from mycelia, and thick-walled mycelia exhibiting irregular lumen, and from *Pseudophacidium* by the flaring phialide collarettes. Based on the *RPB2* phylogeny, *Calvophomopsis* forms a clade sister to *Gloeopycnis protuberans* (Fig. 4.2)

Description: Conidiomata pycnidoid, initially immersed then becoming erumpent, irregularly multilocular, dark brown, opening by irregular tears of the upper layer to reveal hymenium; wall 1–2 layers of textura intricata to textura angularis; Conidiophores arising from inner layer of conidioma, reduced to conidiogenous cells or sparsely branched, septate. Conidiogenous cells discrete, at times integrated, cylindrical

to ampulliform, smooth with conspicuous periclinal thickening or percurrent proliferation at apex. Conidia aseptate, hyaline to subhyaline, smooth, thin-walled, broadly naviculate to ellipsoidal, lacking appendages, exuded in droplets.

Type species: Calvophomopsis rubensicola J.B. Tanney.

Calvophomopsis rubensicola J.B. Tanney, sp. nov. Fig. 4.6.

Mycobank: MBXXXXXX

Typification. Canada: New Brunswick, Alma, Fundy National Park, Coppermine Trail, 45.5493 -65.01878, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 16 Jul 2014, J.B. Tanney NB-RS9-9D (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC 250729.

Etymology: Named for its host, *Picea rubens*.

Colonies 34–39 mm diam after 10 d in the dark at 20 °C on MEA; flat, very sparse hyaline upright aerial mycelia in center, margin diffuse, flat, wide, plumose, greyish yellow (2B4) to hyaline, surface and reverse olive yellow to yellow (2D8–2F8). Exudates and soluble pigments absent.

Conidiomata in MEA pycnidoid, gregarious to caespitose, brownish grey (9F2) to black, up to 1000 µm diam; irregularly multilocular or convoluted; wall 20–60 µm thick, dark brown (8F8), textura intricata, pseudoparenchymatous, gelatinized; cells of outer layer brown, thick-walled, textura intricata, textura globulosa, or textura angularis; cells of inner layer hyaline to subhyaline; no definite ostiole present, dehiscent by irregular

tearing of the covering layer. Conidiophores arising from inner hyaline layers of locule, hyaline, smooth, simple or sparsely branching, up to 20 µm long, 2.5–3 µm diam, invested in mucus. Conidiogenous cells phialidic, discrete, occasionally integrated, cylindrical to ampulliform, conspicuous periclinal thickening or percurrent proliferation, hyaline to subhyaline, smooth, (6.5–)8–11(–12) × 2–2.5 µm. Conidia aseptate, hyaline to subhyaline, smooth, thin-walled, broadly naviculate to ellipsoidal, tapering at ends, apex broadly rounded, base more acutely rounded or narrowly truncate, 5–6 × 3(–3.5) µm, appendages not observed, containing 2(–3) guttules (1.5–)2(–2.5) µm diam, exuded in pale yellow to pale orange (4A3–5A3) droplets.

Cardinal temperatures: Range 5–30 °C, optimum 20–25 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: see Supplementary Table 4.1.

Notes: *Calvophomopsis rubensicola* is phylogenetically distinct but morphologically similar to *Allantophomopsiella*, *Phacidiopycnis*, and *Pseudophacidium* spp.

Calvophomopsis rubensicola conidia are similar to those of *Allantophomopsiella pseudotsugae* (5–8 × 2.5–3 µm, ellipsoid to fusiform; Crous et al. 2014b) but lack mucoid apical appendages. *Phacidiopycnis* spp. exhibit conidia budding from hyphae and microcyclic conidiogenesis, which were never observed in *C. rubensicola*.

Calvophomopsis rubensicola conidia are morphologically similar to those described from some strains of *Pseudophacidium piceae* (e.g.: M 7055: $5.7 \pm 1.3 \times 3.1 \pm 0.6 \mu\text{m}$; Egger 1968), but strains lack the morphological variability described in *P. piceae*. Conidia of *Pseudophacidium diselmae*, isolated from *Diselma archeri* stem cankers in Australia, are similar ($5\text{--}7 \times 2\text{--}3 \mu\text{m}$, ellipsoidal) but borne on shorter conidiogenous cells, $3\text{--}4 \times 2\text{--}2.5 \mu\text{m}$ (Yuan et al. 2000). *Calvophomopsis rubensicola* differs from *Gloeopycnis rubensicola* by conidia morphology, lack of conidia budding from hyphae, and ITS and *RPB2* sequences: ITS sequences between the two ex-type strains differ by 8 bp [identities = 578/586 (99%), gaps = 1/586 (0%)] and *RPB2* sequences differ by 48 bp [Identities = 636/684 (93%), gaps = 0/684(0%)].

Gloeopycnis J.B. Tanney gen. nov.

MycoBank: MBXXXXXX

Etymology: From Latin gloeo, sticky, named for the conidia produced in gloeoid masses from pycnidoid conidiomata.

Diagnosis: Differs from *Allantophomopsis* by the lack of conidial appendages and annellations of the conidiogenous cells, from *Strasseria* by lack of both conidial basal cellular appendages and mucoid apical appendages, from *Allantophomopsiella* by the lack of conidia with mucoid apical appendages, and from *Phacidiopycnis* by the absence of microcyclic conidiogenesis. Morphologically similar to *Phacidiopycnis* and *Pseudophacidium* but phylogenetically distinct based on ITS sequences.

Description: Conidiomata pycnidoid, superficial or immersed then erumpent, irregularly multilocular; dark brown, no definite ostiole present, dehiscent by irregular tearing of the covering layer; wall of 1–2 layers of subhyaline to dark brown textura intricata; conidiophores arising from inner layers of conidioma, reduced to conidiogenous cells or sparsely branched, invested in mucous. Conidiogenous cells phialidic, discrete or integrated, cylindrical to lageniform or ampulliform, conspicuous periclinal thickening and flaring collarete sometimes observed, hyaline to subhyaline, smooth. Conidia morphologically diverse, aseptate, hyaline to subhyaline, smooth or with prominent tubercular protuberances, thin- or thick-walled, fusiform-cylindrical to fusiform-clavate or broadly naviculate to ellipsoidal, appendages absent, exuded droplets.

Type species: *Gloeopycnis protuberans* J.B. Tanney.

Gloeopycnis protuberans J.B. Tanney, sp. nov. Fig. 4.7.

Mycobank: MBXXXXXX

Typification. Canada: New Brunswick, Alma, Fundy National Park, Coppermine Trail, 45.5493 -65.01878, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 16 Jul 2014, *J.B. Tanney RS10-9I* (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC 250727.

Etymology: Named for the protuberances observed on some conidia

Colonies 19–22 mm diam after 10 d in the dark at 20 °C on MEA; flat, sparse hyaline aerial mycelia, margin diffuse, flat, wide, uneven, gnawed, hyaline; surface

white, olive yellow (3D6) toward centre; reverse white, greyish yellow (4C6) toward centre. Exudates and soluble pigments absent. Numerous immature pycnidia in centre.

Conidiomata in MEA pycnidiod, superficial or immersed and later becoming erumpent, abundant, gregarious to caespitose, brownish grey (9F2) to black, glabrous or pubescent with hyaline to greyish yellow (4C3) hyphae, up to 500 µm diam; irregularly multilocular; wall 20–60 µm thick, dark brown (8F8), gelatinized; cells of outer layer brown, thick-walled, *textura intricata*; cells of inner layer hyaline to subhyaline; no definite ostiole present, dehiscent by irregular tearing of the covering layer.

Conidiophores arising from inner hyaline layers of locule, hyaline, smooth, frequently reduced to conidiogenous cells or sparsely branched, up to 20 µm long, 2.5–3 µm diam, invested in mucus. Conidiogenous cells phialidic, discrete or integrated, cylindrical to lageniform or ampulliform, conspicuous periclinal thickening and flaring collaette sometimes observed, hyaline to subhyaline, smooth, (5–)6.5–10.5(–14) × (2–)2.5–3.5(–4.5) µm. Conidia morphologically diverse, consisting of aseptate, hyaline to subhyaline, smooth, thin-walled, fusiform-cylindrical to fusiform-clavate or broadly naviculate to ellipsoidal, tapering at ends, apex broadly rounded, base more acutely rounded or narrowly truncate, some conidia thicker-walled, greyish yellow (4C5), with prominent tubercular protuberances, 7–9(–10) × (2.5–)3–3.5 µm, appendages not observed; containing 2–3(–4) guttules 2–2.5 µm diam, exuded in pale yellow to light orange (4A3–5A5) droplets.

On MEA cultures, conidia sometimes produced externally in hyaline slimy accumulations on the agar surface from coils of swollen or variable hyphae. These conidia are morphologically quite variable and are frequently malformed or apparently devoid of cytoplasm with the cell wall ruptured. Sparse conspicuous phialides are present within these hyphal masses and conidial budding was sometimes observed. On AJA, conidial budding from hyphae is more evident although conidia are less abundantly produced. Budding conidia on AJA (3.5–)4.5–8.5(–12.5) × (2–)2.5–4.5(–6) μm, pyriform to ellipsoid or fusiform-ellipsoidal.

Cardinal temperatures: Range 5–30 °C, optimum 20–25 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: see Supplementary Table 4.1.

Notes: *Gloeopycnis protuberans* is phylogenetically distinct but morphologically similar to other Phacidiales species lacking appendages, including *Phacidiopycnis* and *Pseudophacidium* spp. *Phacidiopycnis boycei* (= *Potebniamyces balsamicola* var. *boycei*), described from *Abies lasiocarpa* in British Columbia, produces angular, fusoid-triangular to trapezoidal conidia similar to the malformed conidia observed in *G. protuberans* cultures on MEA, however *P. boycei* does not produce fusiform-cylindrical conidia and the colony morphology on MEA, colourless at first then becoming greenish and

eventually turning dark brown, distinguishes it from *G. protuberans* (Funk 1970). The phacidiopycnis state of *Potebniomyces gallicola* consists of shorter (6–7 × 3–4 µm) ellipsoidal or inequilateral conidia produced from diminutive (2–3 µm long) simple phialides (Funk and Smith 1981). *Pseudophacidium garmanii*, described from *Picea engelmannii* and *P. glauca* in British Columbia, Canada, differs from *G. protuberans* by its strongly pyriform, wider conidia (8–12 × 4–6 µm) lacking protuberances and the microconidia or globose structures produced on the apices of conidiophores in conidiomata (Funk 1980b). *Pseudophacidium gaeumannii* conidia are similar in dimension to those of *G. protuberans* (7.5–10.5 × 1.5–3 µm), however they are narrowly cylindrical with rounded ends (Egger 1968). Similar to *G. protuberans*, *Pseudophacidium piceae* conidia are quite variable, ranging from 4.5–12 × 1.5–7.5 µm with approximately 1/3 of conidia in pycnidia being large and more-or-less cylindrical and the remaining smaller and ellipsoidal (Egger 1968). Based on ITS and *RPB2* sequences, *Gloeopycnis protuberans* is phylogenetically distant from specimens of *Pseudophacidium ledi* and *P. piceae*.

Cornibusella J.B. Tanney gen. nov.

MycoBank: MBXXXXXX

Etymology: From Latin cornibus, horn, named for the horn-like stromatic projections.

Cornibusella ungulata J.B. Tanney, sp. nov. Fig. 4.8.

Mycobank: MBXXXXXX

Typification. Canada, New Brunswick, Albert County, Alma, Fundy National Park, East Branch trail, 45.64335 -65.11563, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 25 Sep 2013, J.B. Tanney NB-392-3M (holotype DAOM 628553). Ex-type culture DAOMC 250730.

Etymology: From Latin ungula, hoof, named for the hoof-like cloven apices of the stromatic projections.

Colonies 25–27 mm diam after 10 d in the dark at 20 °C on MEA; flat to slightly convex in centre, sparse to moderate cottony hyaline aerial mycelia in center, margin diffuse, flat, wide, circular to somewhat sinuate, white; surface and reverse olive brown (4F5). Exudates and soluble pigments absent.

Stromatic projections occurring solitarily or in dense clusters, up to 10 mm tall, 1–2 mm diam, brownish grey (7F2), surface smooth or pruinose with brownish grey to greyish brown (5C2–5D3) hyphae, simple or branching, occasionally anastomosing, proliferating percurrently resulting in cleft-like swellings along the length giving a knobby appearance, apex rounded to truncate or cloven. Outer layer pseudoparenchymatous, comprised of melanised densely packed, thick-walled textura angularis with hyphal wefts toward surface, inner layer pale comprised of pale brown

textura globulosa to textura angularis, conspicuous lumina 15-45 µm present throughout tissue.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: see Supplementary Table 4.1.

Notes: *Cornibusella ungulata* strains are readily identified by the abundant stromatic projections in culture (Fig. 4.8). The stromatic projections are morphologically consistent and while it is predicted that pycnidoid conidiomata should develop within these formations, conidia or conidiomata have never been observed despite prolonged incubation (3 yr) on a variety of media, substrates, and growth conditions. Lagerberg et al. (1928) described a type of *Allantophomopsiella pseudotsugae* strain (as *Discula pinicola* var. *mammosa*) that was distinct and readily identifiable by the production of black, firm, well-delineated, pulvilliform sclerotia up to 2 mm diam with finely capillose surfaces. The var. *mammosa* sclerotia or stromatous structures were usually sterile although in some strains pycnidia were occasionally produced. The stromatic projections produced by *Cornibusella ungulata* are morphologically different than those described in the var. *mammosa* and ITS sequences of *C. ungulata* are distinct from *A. pseudotsugae*. *Cornibusella ungulata* diam growth becomes restricted at 25 °C.

DISCUSSION

In this study, seven Phacidiaceae species were recovered as endophytes from healthy *Picea rubens* needles. Reports of Phacidiaceae endophytes of conifers are not especially common (e.g.: Sokolski et al. 2007, Koukol et al. 2012, Prihatini et al. 2015b), although unidentified sequences accessioned in GenBank suggest their existence in healthy conifer needles is more prevalent than reported (e.g.: Stefani and Bérubé 2006, U'Ren et al. 2010, Osono and Hirose 2011). Reports of Phacidiaceae species from *Picea* were previously limited to *Allantophomopsiella pseudotsugae*, *Ceuthospora pithyophila*, *Darkera parca*, *D. picea*, *Phacidium abietinum*, *Phacidium lacerum*, *Pseudophacidium garmanii*, *P. ledi*, *P. piceae*, and *Strasseria geniculata* (Farr and Rossman 2016).

In this chapter, three novel monotypic genera were erected to accommodate endophyte species that were phylogenetically and morphologically distinct and consequently could not be satisfactorily placed within existing genera. *Calvophomopsis rubensicola* and *Gloeopycnis protuberans* produce conidia that lack appendages, a character shared by *Phacidiopycnis* and *Pseudophacidium* spp. *Phacidiopycnis* spp. are characterized by morphologically diverse conidia, microcyclic conidiogenesis, conidia budding directly from hyphae in a manner reminiscent of *Aureobasidium pullulans*, and the presence of abnormal hyphae with irregular wall thickenings causing variable lumen diameter (Potebnia 1912, Funk 1970). These characters were absent in *Calvophomopsis rubensicola*; however, *Gloeopycnis protuberans* exhibits some characters attributable to *Phacidiopycnis*, such as variable conidia morphology including the presence of

irregularly shaped conidia similar to those of *Potebniamyces balsamicola* (as *Potebniamyces balsamicola* var. *boycei*; Funk 1970) and the production of conidia from both pycnidoid conidiomata and budding directly from hyphae. *Calvophomopsis rubensicola* is genetically distinct from the clade containing *Allantophomopsiella pseudotsugae*, *Phacidiopycnis washingtonensis*, *Potebniamyces pyri*, all of which exhibit microcyclic conidiation and budding, and several unidentified ITS sequences of purported *Phacidiopycnis* spp. (Fig. 4.1), supporting its distinction and indicating conidial budding may be present throughout the Phacidiales lineage. Conidial budding was more conspicuous in *C. rubensicola* cultures grown on AJA than other media; hyaline droplets of conidia produced in this manner were observed from hyphal coils on MEA but conidiogenesis was less evident and conidia appeared malformed. *Gloeopycnis protuberans* and *C. rubensicola* were the most frequently isolated Phacidiaaceae endophytes from *Picea rubens* in this study. *Cornibusella unguolata* was sterile in culture but consistently identifiable by its large stromatic projections. Pycnidoid conidiomata should conceivably develop within these structures; however conidiomata or conidia were never observed despite many attempts to induce their formation for three years. *Cornibusella unguolata* is phylogenetically distinct and closest related to *Pseudophacidium ledi* (DAOM 129880) based on the ITS phylogeny (although weakly supported; P.P. = 0.68). Previous workers described two distinct cultural forms of *Allantophomopsiella pseudotsugae*, a less common type characterized by appressed growth, scant aerial mycelia, and the rapid formation of numerous pycnidia and a more common form or “stromatiferous type”, characterized by the formation of pulvinate,

capillose, sclerotium-like hyphal growths up to 4 mm in diameter, which typically remained sterile but occasionally produced pycnidial locules after prolonged incubation. Lagerberg et al. (1928) considered the stromatiferous type as a variety of *Allantophomopsiella coniferarum* (as *Discula pinicola* var. *mammosa*) and noted that conidia were frequently found in slides from one-month-old cultures; however, the authors were unable to determine their development. These conidia were morphologically different from those produced in pycnidia, suggesting they may result from conidial budding. Lagerberg et al. (1928) observed that in the majority of stromatous formations, their morphology did not change and they remained infertile indefinitely. In some strains, pycnidia were occasionally found after an incubation period of 6 mo or longer, revealed by the presence of a flesh-coloured droplet containing conidia. *Cornibusella unguolata* is considered distinct from the “*Discula pinicola* var. *mammosa*” strains based on morphological differences of the stromatic projections and its phylogenetic distinction from *Allantophomopsiella pseudotsugae*. It is unclear if strains of the *Allantophomopsiella pseudotsugae* “var. *mammosa*” variety have been sequenced, but determining if these strains are in fact conspecific with *A. pseudotsugae* is of some interest.

Four *Strasseria geniculata* strains were isolated from *Picea rubens* needles (Fig. 4.9). *Strasseria geniculata* can be readily identified based on morphological characters, namely the conidia bearing a distinct tubular basal appendage delineated from the main conidial body by a septum and a funnel-shaped mucoid apical appendage. *Strasseria geniculata* is common on fallen or necrotic *Picea abies* and *Pinus radiata* needles (Evans

and Oleas 1983, Przybył et al. 2008, Prihatini et al. 2015a) and is associated with spring needle cast of *P. radiata* in Tasmania, although its pathogenicity remains unsubstantiated (Podger and Wardlaw 1990). Durán et al. (2008) frequently isolated *S. geniculata* from *Pinus radiata* needles showing early symptoms of “Daño Foliar del Pino” disease and observed *S. geniculata* pycnidia at the bases of symptomatic needles. However, the authors reported that *S. geniculata* inoculation studies with young and old *P. radiata* plants did not result in disease symptoms. Including its association with conifer foliage, Parmelee and Cauchon (1979) observed *S. geniculata* pycnidia on branches and twigs of *Abies*, *Picea*, and *Pinus* and the bark of *Picea glauca*, and noted it was the dominant fungus associated with small (1–2 mm diam) branch cankers on *Pinus sylvestris*. *Strasseria geniculata* is also implicated in secondary blue stain of fallen trees and sawn timber (Butin 1995, McCarthy et al. 2010). *Strasseria geniculata* is reported from a wide variety of host plant families, including Ericaceae (e.g.: *Pyrola* and *Vaccinium*), Rosaceae (e.g.: *Crataegus*, *Malus*, *Prunus*, *Pyrus*), and Lycopodiaceae (*Diphasiastrum complanatum*) (Sutton 1980, Nag Raj 1983). It is one of the causal agents of black rot in commercial *Vaccinium* crops (Oudemans et al. 1998).

Darkera parca is reported as a common endophyte from healthy *Picea* needles in both Europe and North America (Sieber 1988, Müller and Hallaksela 1998, Lorenzi et al. 2006b, Sokolski et al. 2007). For example, Sieber (1988) and Sokolski et al. (2007) reported *D. parca* as the second most frequently isolated endophytes from *Picea abies* in Switzerland and *P. mariana* in Quebec (Canada), respectively. Heiniger and Schmid (1989) isolated *Darkera parca* cultures from 32% of 199 surface-sterilized green *Picea*

abies needles and in some sites observed *D. parca* conidiomata on 50% of all shed brown needles. Although Whitney et al. (1975) observed *D. parca* associated with damaged areas on living needles, *Darkera parca* is probably an endophyte or weak opportunistic pathogen that sporulates following needle senescence resulting from stress or age (Sieber 1988, Karadžić 1998). In contrast, *Darkera durmitorensis* is an aggressive pathogen that colonizes *Abies alba* needles of all ages and, in conjunction with other fungi, may lead to tree death (Karadžić 1998, Karadžić 2003). The sexual states of *Darkera* species bear several morphological features that are uncharacteristic of the Phacidiaceae, notably a covering layer composed of textura epidermoidea and inamyloid asci with thin-walled apices that irregularly tear to release the ascospores (Whitney et al. 1975, Dicosmo et al. 1984). *Darkera abietis* and *D. parca* usually bear eight-spored asci, however some asci may contain only four–seven ascospores (Whitney et al. 1975).

The species limits of *Darkera* species associated with *Picea* needles are unclear. Whitney et al. (1975) described the type species, *Darkera parca*, and designated the sexual state holotype from *Picea glauca* needles from Alberta, Canada. The asexual state holotype (as *Tiarosporella abietis*) is from *Picea abies* needles in England (as *Sphaeropsis parca* Berk. & Broome 1850). Nag Raj (1993) examined the *D. parca* holotype (DAOM 145413b, as *Tiarosporella parca*) and recorded larger conidial dimensions than those observed by Whitney et al. (1975): (29–)35–43 × 9–12 µm vs. (20–)23–40 × 4–6(–7) µm. Strains identified by Crous et al. (2015b) as *Darkera parca* from healthy *Picea obovata* needles in Siberia share conidial dimensions closer to those

described by Whitney et al. (1975) than Nag Raj (1993). Crous et al. (2015b) described *Darkera picea* from *Picea abies* needles in Finland, Norway, and Switzerland; *D. picea* is characterized by large conidia with dimensions almost exactly those of the *Darkera* cf. *parca* strains isolated in this study. ITS sequences of *Darkera picea* (ex-type CBS 138576) and the New Brunswick *Darkera* cf. *parca* strains are similar [identities = 540/544 (99%), gaps = 1/544 (0%)], however the *TEF1α* sequences are more divergent [identities = 383/395(97%), gaps = 11/395(2%)] because of an 11 bp intron deletion in the Canadian strains (Fig. 4.3). Intraspecific variation of *TEF1α* sequences among *Darkera picea* strains is 1–3 bp. ITS and *TEF1α* sequences originating from Canadian *D. parca* holotype material (DAOM 145413b, as *Tiarosporella parca*) distinguish it from the Siberian *D. parca* strains [e.g. CPC 23903, ITS: identities = 559/564 (99%), gaps = 1/564(0%); *TEF1α*: identities = 348/355 (98%), gaps = 4/355 (1%)], *Darkera picea* [ITS: identities = 557/565 (99%), gaps = 2/565 (0%); *TEF1α*: identities = 352/357 (99%), gaps = 2/357 (0%)], and the New Brunswick *Darkera* sp. strains [ITS: identities = 529/536 (98%), gaps = 1/536 (0%); *TEF1α*: identities = 353/370 (95%), gaps = 13/370 (97%)]. Numerous ITS sequences accessioned into GenBank as unidentified endophytes isolated from *Picea glauca* (Alaska, U.S.A.) and *Picea mariana* (Quebec, Canada) are actually species of *Darkera*, most likely conspecific with the New Brunswick species (Fig. 4.10). Based on sequence and morphological data, *Darkera* associated with *Picea* needles could be considered a single species (*D. parca*) occurring along temperate and boreal zones worldwide, with intraspecific variation reflecting biogeographic and host differences. Using combined fatty acid and sterol (FAST) profiles and randomly amplified

microsatellite (RAMS) markers, *D. parca* from *Picea abies* in Finland, Norway, and Switzerland could be separated into three non-overlapping populations based on geographic origin; based on similarity of RAMS patterns and morphological characters, the authors concluded that *T. parca* is probably a single species (Müller and Hantula 1998). Alternatively, ITS and *TEF1 α* sequences suggest the presence of a complex of phylogenetically distinct species that warrants further study. The description of the New Brunswick *Darkera* strains as a novel species is deferred because of a lack of morphological differences or clear host/biogeographical pattern and an overall insufficient sampling of *Picea*-associated *Darkera* spp. in North America and worldwide (Fig. 4.11). Additionally, the connection between the two *Darkera parca* holotypes must be resolved; is the *D. parca* holotype (*Picea glauca*, Alberta, Canada, DAOM 145413a) conspecific with the *Tiarosporella parca* holotype (*Picea abies*, Wiltshire, England, KEW)?

ITS sequences were generated from herbarium specimens of *Allantophomopsis cytispora* (DAOM 211694), *Phacidium phacidioides* (as *Ceuthospora phacidioides*; DAOM 109673, 56835), *Pseudophacidium ledi* (DAOM 129880), *P. piceae* (DAOM 129876, 129879), *Allantophomopsiella pseudotsugae* (as *Potebniamyces coniferarum*; DAOM 129858, 129883), and type specimens of *Darkera parca* (DAOM 145413) and *Phacidium lunatum* (DAOM 180582). The *Pseudophacidium piceae* DAOM specimens share identical ITS sequences with *Pseudophacidium ledi* CBS 377.59, a strain isolated from *Picea abies* in Switzerland that is probably misidentified. A *Pseudophacidium ledi* (DAOM 129880) specimen on *Rhododendron groenlandicum* from Quebec, Canada is

distinct from *P. ledi* CBS 377.59 and the *P. piceae* specimens. Egger (1968) noted that *Pseudophacidium* species were difficult to distinguish because of extreme intraspecific morphological variability and considered the plant host of importance: *P. ledi* is associated with Ericaceae while *P. piceae* is associated with *Picea*. Therefore CBS 377.59 is probably *P. piceae* when accounting the host (*P. abies*) and broad range of conidia dimensions reported by Crous et al. (2014b). Sequences of additional *P. ledi* strains are required to determine if host specificity is a good criterion for delineating *P. ledi* and *P. piceae*. *Pseudophacidium piceae* is a reported pathogen of *Abies balsamea*, *Picea* spp., and *Pinus sylvestris* while inoculation tests for pathogenicity were negative for *P. ledi*, providing some biological support for their distinctiveness (Smerlis 1969).

ITS sequences of the two Canadian *Allantophomopsiella pseudotsugae* (as *Potebniomyces coniferarum*) DAOM specimens share 100% similarity with two *Allantophomopsiella pseudotsugae* strains (CBS 288.37, gb KJ663824; WPF-21-12A, gb KT000147) and 99% similarity with sequences of isolates identified as *Potebniomyces pyri* [e.g.: AY606256; identities = 552/559 (99%), gaps = 1/559 (0%)], *Phacidiopycnis washintonensis* [e.g.: KP759280; identities = 552/559 (99%), gaps = 1/559 (0%); not actually conspecific with *P. washingtonensis*], and the ex-epityes of *Allantophomopsis cytisporea* [CBS 140061, gb NR132921; identities = 551/557 (99%), gaps = 1/557 (0%)] and *A. lunata* [NR132922; identities = 539/546 (99%), gaps = 2/546 (0%)].

Allantophomopsiella was recently erected to accommodate *A. pseudotsugae*, a facultative pathogen of *Larix*, *Picea*, *Pinus*, and *Pseudotsuga*, associated with wounds resulting from pruning, frost, and browsing damage (Uotila 1990, Crous et al. 2014b,

Farr and Rossman 2016). The fungus actively colonizes host phloem during the cold dormant season and causes significant cankers, stem girdling, and shoot dieback (Hood and Sandberg 1985, Uotila 1990, Wainhouse et al. 1997, Crous et al. 2014b). Crous et al. (2014b) distinguished *Allantophomopsiella* from *Allantophomopsis* based on the lack of percurrent proliferations of the conidiogenous cells and the conidia shape (inequilaterally fusiform or naviculate). *Allantophomopsiella* is closely related to *Phacidiopycnis* (= *Potebniamyces*; Johnston et al. 2014), however *Phacidiopycnis* conidia lack appendages. Hahn (1957) originally described *Allantophopsiella pseudotsugae* as *Phacidiella coniferarum* and described its phacidiopycnis anamorph consisting of conidia that are often variable in size and shape and notably exhibit microcyclic conidiogenesis or bud directly from mycelia, consistent characters of other described *Phacidiopycnis* species (Smerlis 1962, Liu and Xiao 2004, Xiao et al. 2005). Authors have also noted that *Phacidiopycnis* mycelia are sometimes thick-walled with a conspicuously irregular lumen, a feature included in the original description of *A. pseudotsugae* (Potebnia 1912, Brooks 1928, Hahn 1957, Funk 1970). *Phacidiopycnis* is probably the asexual state of *Potebniamyces*; however this relationship must be verified by recollecting the type species, *Phacidiopycnis malorum* (Crous et al. 2014b). The identity of *P. malorum* s.s. must also be determined; von Höhnelt (1917) considered *Phacidiopycnis malorum* synonymous with *Phacidiopycnis pyri* (as *Cytospora pyri*) and Weindlmayr (1964) later synonymized *P. malorum* with *P. pyri*.

Apostrasseria was described for *Ceuthospora lunata*, *Strasseria lycopodina*, and three undescribed coelomycetes displaying lunate to fusiforme or naviculate conidia

with apical or polar funnel-shaped mucoid appendages borne from phialides or annelides (Nag Raj 1983). The *Apostrasseria lunata* concept was rather broad, with conidia ranging from $5\text{--}13 \times 2\text{--}3\text{--}3.5$ μm and a noticeable discrepancy between the conidia dimensions of the designated *A. lunata* type ($6\text{--}9 \times 2\text{--}3.5$ μm ; BPI 365950, from fallen leaves of *Vaccinium macrocarpon*) and the original *Strasseria lycopodina* type specimen later synonymized under *A. lunata* ($8\text{--}12 \times 2\text{--}2.5$ μm ; from *Lycopodium complanatum*) (Höhnel 1909, Nag Raj 1983). A new combination was later made for *Allantophomopsis lycopodina* (= *S. lycopodina*) to maintain its distinction (Carris 1990). The type species of *Apostrasseria*, *A. lunata*, was later synonymized with *Allantophomopsis cytispora* by Carris (1990), who observed both type specimens and felt the degree of locular partitioning was not an adequate justification for maintain the two species as separate taxa, a move later supported by Nag Raj (1993). Recently, Crous et al. (2015a) made the new combination *Allantophomopsis lunata*.

Crous et al. (2015a) epitypified *Allantophomopsis cytispora* and *A. lunata*.

There is one bp difference between the ITS sequences generated from the *Phacidium lunatum* holotype (DAOM 180582 as "*P. lunatus*", purported *A. lunata* sexual state, from fallen leaves of *Gaultheria procumbens*, New York, U.S.A., Coll. F. DiCosmo, Det. T.R. Nag Raj, Sept. 1976) in this study and the *Allantophomopsis cytispora* ex-epitype (CPC 24977; from *Oxycoccus macrocarpus* berries) designated by Crous et al. (2015a) [NR132921; identities = 477/478 (99%), gaps = 0/478 (0%)]. The ex-epitype culture (CPC 24977) description depicts conidia dimensions [$(5\text{--})6\text{--}6.5\text{--}7 \times 2\text{--}2.5$ μm] that are somewhat more similar to those described for the type specimen of *Allantophomopsis*

lunata (as *Phacidium lunatum* DAOM 180582; $7-9 \times 2-2.5 \mu\text{m}$) than those of the *Allantophomopsis cytispora* type [(5-)7-10(-12) \times 2-3.5 μm] by (Carris 1990). Based on morphological similarities, it is possible that the *Phacidium lunatum* asexual state described by DiCosmo et al. (1983, 1984) is actually *Allantophomopsis cytispora*. Furthermore, an *Allantophomopsis cytispora* specimen (DAOM 211694) shares a 100% similar ITS sequence [NR132922; identities = 477/477 (100%), gaps = 0/477 (0%)] with the *Allantophomopsis lunata* epitype designated by Crous et al. (2015a) (CBS H-22266). A re-examination of *A. cytispora* DAOM 211694 and the *P. lunatus* holotype DAOM 180582 shows virtually identical conidia dimensions: (7-)7.5-8.5(-9.5) \times (2-)2.5-3 μm (*A. cytispora*) vs. (6.5-)7.5-8.5(-9.5) \times 2-2.5(-3) μm (*P. lunatus*). This discrepancy is probably a result of misidentification of DAOM 211694; however, it highlights the issues identifying *A. cytispora* and *A. lunata*, especially when both may occur on the same host in the same locality. In consideration of the necessity for authenticated reference sequences to assist species delineation and identification, as well as the importance of living ex-epitype strains, the epitypes selected by Crous et al. (2015a) should probably be favoured despite possible discrepancies with their identifications. Crous et al. (2015a) opined that *A. cytispora* is probably a species complex and futures isolations of “*A. cytispora*” isolates from different hosts will prove to be phylogenetically distinct. Currently, *Allantophomopsis* is polyphyletic based on sequences of ex-epitype strains for *A. cytispora*, *A. lunata*, and *A. lycopodina* in the ITS phylogeny presented by Crous et al. (2015a).

Increased sampling efforts with corresponding morphological observations and sequence data are necessary to improve our understanding of the biodiversity, ecology, and taxonomy of the Phacidiaceae, a relatively large family that requires a modern treatment. Many species within the Phacidiaceae are reported from wide and overlapping geographic and host ranges, especially among the Ericaceae, Rosaceae, and Pinaceae. The application of the genealogical concordance phylogenetic species recognition concept (GCPSR; Taylor et al. 2000) combined with morphological and biological data should provide answers for species boundaries, but a significant increase in sampling is required to assess intraspecific and interspecific variation. Interspecific and even intergeneric variation of ITS sequences is often low among some Phacidiaceae taxa; for example, ITS sequences of the *Phacidium lacerum* (CBS 761.73) ex-epitype and *P. pseudophacidioides* (CBS 590.69) ex-type cultures differ by only 2 bp and ITS sequences of *Allantophomopsiella pseudotsugae* (CBS 288.37) and *Allantophomopsis cytisporea* (CBS 140061) ex-epitype strains differ by only 6 bp. Species can be more readily delineated with *RPB2* than ITS sequences; for example, *P. faxum* and *P. dicosmosum* share identical ITS sequences but are morphologically distinct with *RPB2* sequences differing by 18 bp. Future work should include culture-based studies with strains accessioned in public culture collections, morphological characterization, ITS barcoding, and multilocus sequence typing using several protein-coding genes (e.g.: *RPB2*, *TEF1 α*) to extend the current sequence dataset.

Molecular phylogenetic studies have placed *Bulgaria inquinans* (formerly Bulgariaceae) within the Phacidiaceae, an unexpected result given the obvious

morphological differences between ascomata of *B. inquinans* and other known Phacidiaceae species (Lantz et al. 2011, Crous et al. 2014b). *Bulgaria inquinans* ascomata are up to 4 cm diam, gelatinous, turbinate, dark brown to black, with a concave hymenium. The ascospores have longitudinal germ slits and are dimorphic, with the top four ascospores being dark brown, thick-walled, and larger than the four hyaline and thin-walled bottom ascospores (Dennis 1977). *Pseudophacidium garmanii* ascomata bear 8-spored asci with irregularly dimorphic ascospores (Funk 1980b) and other members of the Phacidiaceae have asci that sometimes contain an irregular number of ascospores, for example *Allantophomopsiella pseudotsugae*, *Darkera abietis*, and *D. parca* exhibit (4–)8-spored asci (Smerlis 1968, Whitney et al. 1975). The dramatic morphological differences found in *Bulgaria inquinans* compared to other known Phacidiaceae species may be a result of its saprotrophic life history (Wang et al. 2006a). For example, most species within the Cenangiaceae or Rhytismatales are parasitic or endophytic and exhibit highly reduced apothecia, while the outlier saprotrophs *Chlorencoelia* (Cenangiaceae), *Cudonia* and *Spathularia* (Rhytismatales) possess larger, more complex, and morphologically divergent apothecia.

Additional genera placed in the Phacidiales include *Coma* (= *Ascocoma*), *Lophophacidium*, and *Micraspis*. The monotypic genus *Coma* consists of *C. circularis*, a species producing both fuliginous cylindrical conidia bearing flexuous apical and basal appendages and a phialemonium-like spermatial state (Swart 1986, Beilharz and Pascoe 2005). The synanamorphs, (0–)1(–3)-septate ascospores, and thick-walled asci are unlike Phacidiales species. *Lophophacidium* comprises the type species, *L.*

hyperboreum, and *L. dooksii*. *Lophophacidium dooksii* is an obligate pathogen of *Pinus strobus* placed in *Lophophacidium* despite morphological differences including a melanised pseudoparenchymatous stroma continuous along the length of the ascoma versus the discontinuous, two-layered covering stroma of *L. hyperboreum* (Corlett and Shoemaker 1984). Excluding the amyloid ascus apex, the ascoma and asexual state (= *Canavirgella banfieldii*) morphology of *Lophophacidium dooksii* are evocative of the Rhytismataceae, a relationship supported by a recent ITS phylogenetic analysis (Laflamme et al. 2015). DNA sequences of *Lophophacidium hyperboreum* are unavailable at this time; an attempt to sequence a *Lophophacidium hyperboreum* herbarium specimen (DAOM 1788) yielded a good quality 663 bp ITS sequence. BLAST results show the *L. hyperboreum* ITS sequence is closest (but distantly) related to Botryosphaerales species, e.g.: *Saccharata capensis* NR121347; identities = 370/424 (87%), gaps = 17/424 (4%); *Saccharata proteae* EU552145; identities = 548/676 (81%), gaps = 57/67 (4%). Sequencing of additional or fresh collections of *L. hyperboreum* is required to confirm if this sequence is the target species or an unknown co-occurring fungus.

Micraspis contains three species known from conifer trees: *M. strobilina* from *Pinus sylvestris* cones, *M. tetraspora* from decorticated *Picea sitchensis* wood, and the type species *M. acicola*, a needle-associate of *Picea mariana* and *P. rubens* (Darker 1963, Farr and Rossman 2016). Dicosmo et al. (1984) felt Darker's assignment of *Micraspis acicola* to the Phacidiaceae was unwarranted because of atypical morphological characters including the anatomy of the covering layer, thickened in amyloid ascus apex,

3-septate ascospores that produce blastic-phialidic conidia, and its distinct proposed asexual state (= *Periperidium acicola*). In this study, *Micraspis acicola* strains were isolated from conidiomata on dead needles attached to otherwise healthy twigs (Fig. 4.12). The ITS phylogeny confirms the identity of several unknown *Picea rubens* endophytes in this study as *Micraspis acicola* and shows *M. acicola* sister to the Phacidiaceae and Tympanidaceae and (Fig. 4.13). Dicosmo et al. (1984) noted that the ascospore morphology and microcyclic conidiation of *Micraspis acicola* was reminiscent of *Claussenomyces* and *Tympanis* and consequently placed *M. acicola* in the Tympanidaceae (as “Tympaneae”); however, the asexual state of *Micraspis acicola* is unlike any described from known Tympanidaceae species. The ITS phylogeny does not convincingly resolve the placement of *Micraspis acicola* and few sequences of representative Tympanidaceae species and related taxa are available; however morphological and phylogenetic evidence supports the placement of *M. acicola* outside of Phacidiaceae.

Strains representing the species isolated from *Picea rubens* in this study were sent to the J.D. Miller laboratory for characterization of secondary metabolite profiles and bioactivity screening, however the results are currently unavailable. Some novel bioactive natural products are described from Phacidiales spp., for example a purported *Allantophomopsis lycopodina* strain isolated from decaying hardwood produced a novel antifungal γ -lactone, allantofuranone (Schüffler et al. 2009). Allantopyrone A, a novel α -pyrone displaying potent cytotoxicity against HL60 cancer cells, was described from a purported *Allantophomopsis lycopodina* strain isolated from a dead *Fagus* branch

(Shiono et al. 2010). Phacidin, a γ -pyrone isolated from *Potebniomyces balsamicola*, inhibits growth of a variety of species within the Ascomycota, Basidiomycota, Zygomycota, and Oomycota (Chromalveolata) (Funk and McMullan 1974, Sekhom and Funk 1977, Poulton et al. 1979). Several studies have isolated novel bioactive natural products from ascomata and cultures of *Bulgaria inquinans*, a saprotrophic Phacidiales species occurring on decomposing wood. For example, three novel azaphilones, bulgarialactone A(1), B(2), and C(3), were isolated and described from ascomata and cultures of *Bulgaria inquinans*; Bulgarialactone A(1) and B(2) showed moderate antimicrobial activity against *Bacillus brevis*, *B. subtilis*, and *Micrococcus luteus*, moderate cytotoxicity towards murine cells, and moderate toxicity towards *Caenorhabditis elegans* (Stadler et al. 1995). Musso et al. (2010) described bulgarialactone D from *B. inquinans* and reported antiproliferative activity for bulgarialactone B(2) against a panel of human tumor cell lines and a high binding affinity to heat shock protein 90 (Hsp90), a molecular chaperone being investigated as an anticancer drug target. In vitro cytotoxic activity against human cancer cell lines HL60 and K562 was demonstrated for bulgareone A(1) and bulgareone B(2), two anthraquinone dimers described from *B. inquinans* ascomata (Li et al. 2013). These results should stimulate future work investigating the bioactivity and structure of secondary metabolites from Phacidiales strains.

In this study, Phacidiales species were commonly isolated from surface-sterilized, healthy needles of *Picea rubens* from Eastern Canada, indicating their presence as endophytes prior to needle senescence or potential manifestation of disease symptoms.

Little is known about the role that these fungi play in the plant microbiome. The most commonly isolated Phacidiales endophytes were morphologically and phylogenetically distinct, warranting the description of three novel genera and five novel species. The taxonomy of the Phacidiales is still in disarray and future taxonomic efforts in the Phacidiales should focus on DNA barcoding named-but-unsequenced species and the description of novel species to facilitate the identification of species, improve our understanding of species and generic boundaries, and elucidate the biology and ecology of this formerly neglected order.

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AUTHOR CONTRIBUTIONS

JBT conceived and designed the work. JBT acquired the data: provided strains, collections, and sequences from Canadian material; JBT provided the species descriptions, tested all markers and generated sequences (except for sequences provided by Ewald Groenwald), conducted cultural studies and phylogenetic analyses, created all photographic plates, deposited all cultures in DAOMC and specimens in DAOM. JBT and KAS analyzed and interpreted the data and taxonomic considerations. JBT drafted the article; JBT and KAS critically revised the manuscript for content.

Chapter 5: Phylogenetic and taxonomic diversity of culturable endophytes of *Picea* in Eastern Canada

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ABSTRACT

Research interest in endophytic fungi has increased substantially, yet is the current research paradigm capable of addressing fundamental taxonomic questions? More than half of the ca. 30,000 endophyte sequences accessioned into GenBank are unidentified to the family level and this disparity grows every year. A study of *Picea* endophytes in Eastern Canada sought to address these taxonomic issues with a combined approach that involved molecular methods, classical taxonomy, and field work. Surface-sterilized needles resulted in ca. 2,600 axenic endophyte cultures that were primarily sterile. Cultures were characterized using the ITS barcode, clustered in OTUs based on sequence similarity, and groups or individual species were selected for further investigation. The most abundant endophytes included *Lophodermium* cf. *piceae*, *Diaporthe maritima*, *Xylaria ellisii* sp. nov., and Dothideales species. Concentrated field work provided connections between endophyte cultures and their saprotrophic stages on the same host or on different hosts and/or substrates, verified using the ITS barcode. The presence of sporulating material containing taxonomically informative morphological characters facilitated the identification of named-but-unsequenced endophyte species and the description of novel species, including *Tryblidiopsis magnesii*, *Xylaria ellisii*, and the novel monotypic genus and species *Pileospora piceae*. These results provide insight into the complex life histories of conifer needle endophytes and present a straightforward strategy to identify unknown endophyte cultures and sequences by considering the biology and ecology of the whole fungus.

KEYWORDS

foraging ascomycete hypothesis; ITS barcode; *Picea rubens*; Rhytismatales; saprotroph

INTRODUCTION

Increasing research interest in the biodiversity and functional ecology of endophytes has stimulated extensive cultivation and sequencing efforts to investigate endophytes from a wide range of host plants worldwide. However, in such studies most representative endophyte taxa are often unidentified to species or genus (Arnold and Lutzoni 2007, U'Ren et al. 2012, Huang et al. 2016). Endophytes are difficult to identify because of a lack of taxonomically informative cultural characters and a paucity of relevant reference sequences in databases such as NCBI GenBank or UNITE (Kõljalg et al. 2005). Taxonomic instability can also present issues in consistent identification of sequences over time as the quality of sequences databases changes, e.g.: correction of misidentifications and identification of previously unidentified sequences (Arnold and Lutzoni 2007). The growing dependence on sequence-based identification of fungal specimens and cultures and the shift towards biodiversity studies relying on environmental nucleic acid sequences (ENAS) highlights the need for accurately-identified reference sequences connected with voucher specimens and, when feasible, strains in public culture collections.

The identification of endophytes within the current taxonomic system is crucial for meaningful phylogenetic classification, communication of results, connection with previous research, and conceptualizing phylogenetic hypotheses. The inability to confidently name endophytes represented by sterile cultures or ENAS is a significant problem. Sequences can be clustered as OTUs and presented as putative species

hypotheses, leading some authors to advocate a sequence-based classification system (Hibbett and Taylor 2013, Taylor and Hibbett 2013) and even the abandonment of naming fungal species (Money 2013). But what if many unidentified endophytes are actually named-but-unsequenced species or species that can be identified using a field-based approach? If so, adjusting the endophyte research paradigm will yield better results than advocating for the upheaval of the entire current fungal taxonomic and nomenclatural system.

This study is a component of a larger research program investigating bioactive secondary metabolites of conifer endophytes to identify potential biological control agents of the eastern spruce budworm (*Choristoneura fumiferana*), a major forest pest of significant economic importance in Eastern Canada (Miller 2011). Secondary metabolites produced by endophyte strains identified from this ongoing research include insecticidal rugulosin from *Phialocephala scopiformis* (Miller et al. 2002), insecticidal isocoumarins from strains identified endophyte *Conoplea elegantula* (Findlay et al. 1995), insecticidal maleic anhydrides from *Dwayaangam colodena* (Sumarah et al. 2010), antifungal macrolides, pyrenophorol, and sesquiterpenoids from *Lophodermium nitens* (Sumarah et al. 2011, McMullin et al. 2015, Sumarah et al. 2015), antifungal benzofurans and xanthenes from an unidentified Massarinaceae sp. (Richardson et al. 2015), antibiotic and insecticidal metabolites including vermiculin and γ -lactones from an unidentified species (DAOMC 221611; Findlay et al. 2003), and the antifungal griseofulvin from an unidentified *Xylaria* sp. (Richardson et al. 2014).

A persistent problem encountered in this research was the inability to accurately identify endophyte strains isolated from conifer needles. In this study, the taxonomy and phylogeny of culturable endophytes from *Picea glauca*, *P. mariana*, and *P. rubens*, with a focus on *P. rubens*, in New Brunswick, Canada is explored with the goal of identifying strains to species rank and providing evidence of a saprotrophic life history for some common endophytes. This approach addresses the issue of unidentified endophytes by connecting them with cultures, field specimens, and herbarium specimens of named-but-unsequenced or undescribed species using DNA sequences and morphological characters. Some examples of the identification of unknown *Pinus strobus* endophytes using this approach are also provided. These results provide compelling evidence that culturable endophytes can be identified simply by including local fruit body surveys in endophyte studies and considering the overall ecology of the endophytes.

MATERIALS AND METHODS

Isolates

Endophytes were isolated from healthy, asymptomatic *Picea* and *Pinus* needles as described by Tanney et al. (2016a). Trees were located in both managed and natural stands in New Brunswick. Field collections of various conidiomata and ascomata were made in New Brunswick, Ontario, and Quebec, Canada on the basis of their association with the host, inferred phylogenetic relationships with unidentified endophytes, and absence from GenBank. Field collections and cultures were kept in the personal

collection of J.B. Tanney and materials of interest were accessioned into the Canadian National Mycological Herbarium (DAOM) and Canadian Collection of Fungal Cultures (DAOMC). All cultures were maintained on 2% malt extract agar (MEA) at 16 °C under a 12 h light/dark cycle.

Morphology

Vertical sections of conidiomata or ascomata were cut by hand or in 8–12 µm sections using a freezing microtome and mounted in either demineralized water, 85% lactic acid, Lugol's solution with or without 5% KOH pretreatment (Baral 1987), 5% KOH as a clearing agent, lactofuchsin, or cresyl blue. Microscopic measurements were taken from living material, whenever possible, mounted in water and are presented as ranges calculated from the mean \pm standard deviation of each measured value, with outliers in brackets. Colony characters and diameters were recorded for 2- or 4-wk-old cultures inoculated on MEA and incubated at 20 °C. Colony colors were described using the alphanumeric codes of Kornerup and Wanscher (1978). Observations were made using an Olympus BX50 light microscope and micrographs were captured using an Evolution MP Color Camera (Media Cybernetics, Silver Spring, CA, U.S.A.) and Image-Pro Plus v6.0 (Media Cybernetics) or InfinityX-32 camera (Lumenera Corp., Ottawa, ON, Canada) and Infinity Analyze v6.5.2 (Lumenera Corp.) software. Colony macrophotographs were captured with a Nikon Coolpix P5000 (Nikon Inc., Tokyo, Japan) and photographic plates were assembled using Adobe Photoshop v5.5 (Adobe Systems, San Jose, CA, U.S.A.).

DNA isolation, amplification, and sequencing

Total genomic DNA was extracted from 4–12-wk old cultures using the Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) or NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The primers ITS4 and ITS5 (White et al. 1990) were used to amplify and sequence the ITS region. The partial 28S nuc rDNA gene (LSU) region was amplified using the primer pair LR0R and LR5 and sequenced using LR0R, LR3, LR3R, and LR5 (Vilgalys and Hester 1990). Additional loci were amplified and sequenced based on their established usefulness as phylogenetic markers for various taxa, including beta-tubulin (*BenA*; Bt-2a and Bt-2b; Glass and Donaldson 1995) for *Penicillium* (Visagie et al. 2014) and the second largest subunit of ribosomal polymerase II (RPB2-5F and RPB2-7CR; Liu et al. 1999) for *Xylaria* (Tang et al. 2007).

DNA was amplified using a PCR master mix consisting of 0.5 µL 2 mM dNTPs, 0.04 µL 20 µM forward primer, 0.04 µL 20 µM reverse primer, 1 µL 10× Titanium *Taq* buffer (Clontech, Mountain View, CA, U.S.A.), 0.1 µL 50× Titanium *Taq* enzyme (Clontech), 1 µL of DNA template, and 7.32 µL sterile Milli-Q water (Millipore, Bedford, MA, U.S.A.) per reaction. All loci were amplified using the following PCR profile: 95 °C for 3 min, then 35 cycles at 95 °C for 1 min, 56 °C for 45 s, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. PCR products were verified by agarose gel electrophoresis and sequenced with Big Dye Terminator (Applied Biosystems, Foster City, CA, U.S.A.).

For DNA extraction from herbarium specimens, whole or partial ascomata or 2 mm discs of symptomatic plant tissue were removed from dried herbarium specimens (Supplementary Table 5.1) and ground in liquid nitrogen using Axygen polypropylene pestles (PES-15-B-SI, Union City, CA, U.S.A.). Total genomic DNA was extracted using the manufacturer's recommended kit for a Thermo Scientific KingFisher mL magnetic particle processor (VWR, Mississauga, ON, Canada). ITS5 and the ascomycete-specific reverse primer ITS4A (White et al. 1990, Larena et al. 1999) were used to amplify and sequence the ITS region. The previously described PCR profile and PCR master mix were used for amplification, with the addition of 0.5 μ m 20 mg/ml bovine serum albumin (BSA) per reaction.

Sequence contigs were assembled, trimmed, and manually checked using Geneious R6 v6.1.8 (Biomatters, Auckland, New Zealand) and each individual gene dataset aligned using MAFFT v7 (Katoh et al. 2005). The resulting alignments were trimmed and manually checked using BioEdit v7.2.5 (Hall 1999).

Phylogenetic and OTU analyses

ITS sequences for 1,622 *Picea* endophyte strains were aligned with MAFFT, manually checked for ambiguities, and trimmed to identical lengths (Větrovský and Baldrian 2013). Sequences were clustered into operational taxonomic units (OTUs) using the software CD-HIT V4.6.6 (Li and Godzik 2006). A 99% similarity threshold was used for the final analysis (Gazis et al. 2011, Oono et al. 2015), but OTU clustering using

90, 95, 96, 97, 98, and 100% similarity thresholds values were also used to compare number of OTUs by differing similarity threshold.

Select endophyte groups were chosen for further phylogenetic analysis:

Botryosphaeriales, Cenangiaceae, Dothideales, *Hyphodiscus*, Mycosphaerellaceae, *Penicillium* subsect. *Thysanophora*, Phaeomoniellales, Rhytismatales, and Xylariales.

Bayesian analyses were performed for each individual dataset using MrBayes 3.2 (Ronquist and Huelsenbeck 2003) and the most suitable sequence evolution models selected for each analysis based on the optimal Akaike information criterion scores in MrModeltest 2.2.6 (Nylander 2004) (Table 1). For each locus, three independent Markov Chain Monte Carlo (MCMC) samplings were performed with four chains (three heated and one cold) with sampling every 500 generations until the standard deviation of split frequencies reached a value <0.01 . The first 25% of trees were discarded as burn-in and the remaining trees were kept and combined into one consensus tree with 50% majority rule consensus. Convergence was assessed from the three independent runs using AWTY and Tracer v1.6 (Wilgenbusch et al. 2004, Rambaut et al. 2014). Consensus trees were imported into FigTree v1.4.2 (Rambaut 2014) and exported as SVG vector graphics for assembly in Adobe Illustrator 10 (Adobe System, San Jose, CA, U.S.A.).

RESULTS

ITS sequences of the 1,622 *Picea* endophyte strains were clustered into 161 OTUs using a 99% dereplication similarity threshold (Fig. 5.1). The number of OTU clusters increased correspondingly with the similarity threshold (Fig. 5.2). The 99% threshold adequately separated closely-related species sharing similar ITS sequences, for example Phacidiaceae species, however the stringent threshold probably inflated species diversity, for example within Rhytismataceae. The families Rhytismataceae, Mollisiaceae, Xylariaceae, Trichocomaceae, Dothidiaceae, Phacidiaceae, and Phaeomoniellaceae contained the highest number of endophyte OTUs based on ITS sequences. The most abundant species isolated from *Picea* included *Lophodermium* cf. *piceae*, *Rhizosphaera* spp., *Diaporthe maritima*, *Phialocephala scopiformis*, and *Xylaria ellisii* sp. nov. (Fig. 5.3). Several novel species were identified and are described in this or previous chapters, including *Calvophomopsis rubensicola*, *Cornibusella unguata*, *Gloeopycnis protuberans*, *Phacidium dicosmum*, *Phacidium faxum*, *Phialocephala amethystea*, *P. helena*, *P. vermiculata*, *Pileospora piceae*, *Tryblidiopsis magnesii*, and *Xylaria ellisii*.

Unidentified Botryosphaerales strains of a single species, described below as *Pileospora piceae* gen. nov. and sp. nov., were moderately supported [posterior probability value (P.P.) = 0.72] as a sister to the genus *Septorioides* (Septorioidaceae, Botryosphaerales) in the LSU phylogeny (Fig. 5.4). The ITS phylogeny including unidentified endophyte sequences from GenBank strongly supported (P.P. = 0.98)

Pileospora piceae as a sister to *Septorioides* and revealed five unidentified *Picea mariana* endophytes as *P. piceae* (Fig. 5.5). Conidiomata were abundantly produced on MEA and were also detected on dead *Picea rubens* twigs still attached to a living branch (Fig. 5.6).

The ITS phylogeny of Cenangiaceae confirmed the connection of a *Cenangium* sp. (NB-663) specimen collected from a self-bruned *Pinus strobus* branch with two previously unidentifiable *P. strobus* endophytes (DT-7, ON-6C) and several conifer, hepatic, and lichen endophytes (Figs. 5.7, 5.8). *Cenangium* sp. (NB-663) is a sister to *Cenangium ferruginosum* (P.P. = 1). Other Cenangiaceae specimens collected as saprotrophs of decaying plant materials were *Cenangium acuum*, *Chlorencoelia versiformis*, *Heyderia abietis*, and *Hysterostegiella typhae*.

Dothidiaceae endophytes were commonly isolated from *Picea* needles and included four putatively undescribed *Rhizosphaera* species, an unidentified species sister to the *Rhizosphaera-Phaeocryptopus nudus-Scleroconidioma sphagnicola* clade, an unidentified species sister to *Dothiora cannabinae* and *D. europaea* clade, and an unidentified strain related to *Atramixtia aboricola* and *Hormonema macrosporum* (Fig. 5.9). *Celosporium laracicola* and a putatively undescribed *Celosporium* sp. were isolated as *Picea* needle endophytes and form a clade strongly supported (P.P. = 0.97) as a sister to Dothideaceae s.s. Based on the ITS phylogeny, *Rhizosphaera kobayashii* is placed in Aureobasidiaceae and not related to other *Rhizosphaera* species in Dothideaceae.

Strains representing an unidentified *Picea rubens* endophyte were connected with an undescribed *Hyphodiscus* species which produced apothecia on living *Picea rubens* branches (Figs. 5.10, 5.11). The ITS phylogeny placed the *Hyphodiscus* species in a weakly supported (P.P. = 0.59) polytomous clade including the *Soosiella minima* type and several unidentified foliar and root endophytes of conifer and Ericaceae hosts.

Picea rubens endophyte strains of an unidentified Mycosphaerellaceae species formed a clade that is strongly supported (P.P. = 1) as a sister to *Phaeocryptopus gaeumannii* from *Pseudotsuga menziesii* needles, which is in turn sister to *Pallidocercospora* (Fig. 5.12). *Phaeocryptopus gaeumannii* is therefore placed within Mycosphaerellaceae and not *Phaeocryptopus* s.s. (Dothideaceae). Two unidentified *Picea mariana* endophyte sequences from GenBank were conspecific with the Mycosphaerellaceae species detected in this study. Cultures of the Mycosphaerellaceae sp. endophyte were sterile and did not produce conidiomata or ascomata in any of the culture media and conditions after prolonged incubation (2+ yr). Ascomata associated with a Mycosphaerellaceae species were commonly observed on living *Picea rubens* twigs, however they were invariably immature and were not cultured or sequenced (Fig. 5.13).

Throughout summer and autumn in all sampling locations, conidiophores and sclerotia of *Penicillium* subsect. *Thysanophora* species were commonly observed on *Picea* needles that were senescent and still attached to living twigs or dead in the litter layer. Conidiophores were typically concentrated around stomata and also observed in

petioles where needle abscission occurred, and often proliferated from erumpent sclerotia (Fig. 5.14). The *BenA* phylogeny included original sequences derived from strains of endophytes, conidia isolates of needle saprotrophs, and strains isolated from caves in Eastern Canada, as well as sequences from GenBank (Fig. 5.15). The phylogeny revealed four distinct *Penicillium* subsect. *Thysanophora* species were isolated in New Brunswick and that the name *Penicillium glaucoalbidum* (= *Thysanophora penicillioides*) was used for phylogenetically diverse species typically associated with conifer needles.

The ITS phylogeny including species in the family Phaeomoniellaceae (Phaeomoniellales) was a polytomy with overall weak backbone support, showing five distinct clades isolated as *Picea endophytes*, each clade representing a putatively undescribed species (Fig. 5.16). Two of these clades are represented by only one endophyte strain, however 06-251 (*Picea rubens* endophyte) shares identical ITS sequences with several unidentified *Picea mariana* endophyte sequences in GenBank (AY971711, DQ979633, DQ979660, DQ979743, KT289604). Strains examined for two of the species were sterile on all media and growth conditions tested after prolonged incubation (3+ yr).

Rhytismatales endophytes were commonly recovered as *Picea* foliar endophytes and phylogenetically diverse (Fig. 5.17). *Lophodermium cf. piceae* was the most common endophyte and strongly supported (P.P. = 1) as a sister to European collections of *Lophodermium piceae* s.s. (Fig. 5.18). Variability in ITS sequences and colony morphology suggested the presence of cryptic species within *Lophodermium cf. piceae*

from New Brunswick (Fig. 5.1). ITS sequences of unidentified *Picea* endophytes were conspecific or closely related to collections of *Coccomyces irretitus* (Fig. 5.19) and *Tryblidiopsis magnessii* sp. nov., a species described below because of its phylogenetic and morphological distinction from other European and Asian *Tryblidiopsis* species (Fig. 5.20). Unidentified Rhytismataceae endophytes of *Picea mariana* (NB-221-5A and 08-052B) were connected with *Colpoma* cf. *crispum* ascomata collected from dead wood and bark of a fallen *Picea mariana* branch (Fig. 5.21). Many endophyte strains could not be placed in a genus based on ITS sequence data or culture morphology. *Coccomyces strobi* cultures derived from ascospores and conidia originating from specimens collected in Ontario were connected with a previously unidentifiable *Pinus strobus* endophyte (ON-2A). An asexual state of *Coccomyces strobi* was previously unknown and is described in the Taxonomy section (Fig. 5.22). *Therrya fuckelii* on *Pinus resinosa* in Ontario can be readily distinguished from European *Therrya fuckelii* on *Pinus sylvestris* based on ITS sequences (Fig. 5.23). *Anthina flammaea* is placed in Rhytismataceae and ITS sequences suggested distinct species from Canada (NB-763), Europe (CBS 553.93), and Japan (LC098753). ITS sequences obtained for Rhytismataceae species that are absent in GenBank include: *Bifusella pini*, *B. saccata*, *Coccomyces coronatus*, *C. irretitus*, *C. strobi*, *Colpoma crispum*, *Lophodermium aucupariae*, *L. foliicola*, and *Pseudographis pinicola*. Misidentified sequences accessioned in GenBank included: *Lirula macrospora* AF203472 (actually *Tryblidiopsis* cf. *pinastri*), *Meloderma desmazieri* AF203470 (actually *Lophodermium nitens*), and *Tryblidiopsis pinastri* FN868463 (actually *Therrya pini*).

Xylariales endophytes included *Annulohypoxylon multiforme*, *Anthostomella* sp. (NB-382-1E), *Nemania* spp., *Rosellinia* sp., *Xylaria ellisii* sp. nov., and an unidentified species represented by two strains (08-053A/08-053B) which could not be satisfactorily placed within a genus based on the ITS phylogeny (Fig. 5.24). The ITS phylogenies show that Xylariales species are commonly isolated as endophytes or endolichenic fungi from diverse hosts (Figs. 5.24, 5.25). The most commonly isolated Xylariales endophyte was a *Xylaria* species described below as *X. ellisii*. *Xylaria ellisii* was connected with stromata that were commonly found on fallen decaying *Acer saccharum* logs in late summer or autumn in New Brunswick by comparing colony morphology and ITS, LSU, and *RPB2* sequences (Figs. 5.25, 5.26, 5.27).

More than 350 herbarium specimens and new field collections representing over 90 genera and 130 species were included in this study to facilitate identification and/or phylogenetic placement of unidentified endophytes (Supplementary Table 5.1). Some herbarium specimens representing unsequenced species were selected for DNA extraction and ITS barcode sequencing. The success rate for sequencing the ITS barcode of herbarium specimens was generally low, about 26% after excluding short sequences or sequences originating from contaminants or non-target fungi. Common contaminants such as *Candida*, *Malessezia*, and *Simplicillium* may have originated from previous ungloved handling of herbarium specimens or the presence of arthropods, while amplification of some non-target fungi may be attributed to ubiquitous phyllosphere inhabitants or co-occurring endophytes such as *Aureobasidium pullulans* or *Rhizosphaera* spp. The addition of BSA significantly improved amplification of

herbarium specimen DNA, although it also increased amplification of contaminating fungal DNA for some specimens. An example of amplification of non-target DNA from epiphytic or co-occurring fungi included the attempt to sequence *Phacidium arbuti* (DAOM 209147) from *Arbutus menziesii* (Ericaceae) leaves originating from British Columbia, which resulted in a high quality sequence of *Scleroconidioma sphagnicola*, also reported from *Gaultheria shallon* (Ericaceae) leaves in British Columbia (Osono et al. 2008). In some cases, dubious sequences were obtained from species that are unsequenced with an unknown phylogenetic position. For example, *Eupropelella oxycocci* (DAOM 169475 as *Naevia oxycocci*) was placed within the Tympanidaceae and a *Lophophacidium hyperboreum* specimen (DAOM 1788) yielded a good quality 663 bp ITS sequence closest (but distantly) related to Botryosphaerales species, e.g.: *Saccharata capensis* NR121347; identities = 370/424 (87%), gaps = 17/424 (4%). One needle from a paratype specimen of *Lophodermium nitens* (DAOM 109290) yielded a good quality 606 bp long ITS sequence that was a 100% match with *Meloderma desmazieri*, indicating both fungi were present in the specimen. The oldest successfully sequenced specimens were *Lophodermium nitens* (DAOM 109290; coll. 1923) and *Xylaria acutata* (DAOM 203094; coll. 1928).

Sequences were obtained for specimens of genera and species absent or poorly represented by sequences in GenBank, including *Allantophomopsiella pseudotsugae*, *Bifusella linearis*, *B. pini*, *B. saccata*, *Ceuthospora phacidioides*, *Cucurbitaria piceae*, *Darkera parca*, *Grovesiella ledi*, *Hysteronaevia scirpina*, *Galiella rufa*, *Gemmamyces piceae*, *Lophodermium foliicola*, *Lophophacidium dooksii*, *Meloderma desmazieri*,

Mollisia spp., *Neodasyscypha cerina* (DAOM 230366, misidentified as *Haglundia perelegans*), *Niptera discolor*, *Niptera ramincola*, *Nipterella parksii*, *Obtectodiscus aquaticus*, *Pseudophacidium ledi*, *P. piceae*, *Therrya fuckelii*, *Trichobelonium obscurum*, and *Vibrissea* spp. Sequences were also generated from type material of *Darkera parca* (DAOM 145413a), *Obtectodiscus aquaticus* (DAOM 172427), and *Phacidium lunatus* (DAOM 180582).

Picea endophytes connected with sporulating field or herbarium specimens based on ITS sequences and morphology include *Coccomyces irretitus*, *Colpoma crispum*, *Colpoma* sp., *Diaporthe maritima*, *Hyphodiscus* sp., *Lophodermium* cf. *piceae*, *Melanconis alni*, *Micraspis acicola*, *Mollisia novobrunswickia*, *Mollisia* spp., *Phialocephala amethystea*, *P. helena*, *P. nodosa*, *P. piceae*, *P. scopiformis*, *Pileospora piceae*, *Rhizosphaera* spp., *Thysanophora* spp., *Tryblidiopsis magnesii* sp. nov., and *Xylaria ellisii* sp. nov. Sporulating field specimens of *Cenangium* sp. (NB-663) and *Coccomyces strobi* corresponded with unidentified *Pinus strobus* endophytes. Field specimens representing ascomata of Rhytismataceae endophytes were usually found on bark, dead branches, or dead needles still attached to living *Picea* trees (Fig. 5.21) while apothecia of Mollisiaceae endophytes were more frequently recovered from decaying hardwood. Other *Picea* endophytes known to sporulate only on hardwood substrates included *Melanconis alni* and *Xylaria ellisii*.

TAXONOMY

An undocumented asexual state was connected with *Coccomyces strobi* specimens collected in late spring and early summer (Fig. 5.22 D–G). Conidiomata co-occurred with apothecia and identification of cultures generated from conidia as *C. strobi* was verified with ITS sequences. A description of this asexual state is provided here: conidiomata on *Pinus strobus* branches co-occurring with ascomata, pycnidial, lenticular, occurring scattered or gregarious, singly, dark brown to black, subepidermal, erumpent upon maturity, ostiole absent, opening by irregular tear of covering layer, conical to convoluted locule, 300–500 µm diam. Pycnidial wall and covering layer 20–60 µm wide, composed of pale to dark brown textura angularis, thin- to thick-walled, 4–6(–7) × 3–4(–5) µm. Conidiophores forming palisade lining upper and inner pycnidial walls, cylindrical, hyaline, branching 2–3 times, (17–)19–26(–30) × 2–3 µm. Conidiogenous cells smooth, cylindrical to ampuliform, discrete, holoblastic, sympodial to synchronous, 9–11.5(–13) × 2–2.5 µm. Conidia hyaline, smooth, aseptate, cylindrical, straight to curved, apices rounded, (7–)8–12(–22) × (1–)1.5(–2) µm.

Pileospora J.B. Tanney, gen. nov.

Mycobank MB XXXXXX

Etymology: *Pileo* (L.) = hat, *spora* (L.) = spore; referring to the mucoid apical appendages of the conidia.

Conidiomata pycnidial, dark brown to black, unilocular, subglobose to globose, ostiolate; wall consisting of 2–3 layers of dark brown textura angularis, exuding conidial mass in droplet. Paraphyses intermingled among conidiophores, hyaline, cylindrical, aseptate. Conidiophores reduced to conidiogenous cells or sparingly branched. Conidiogenous cells lining the inner pycnidial wall, hyaline, smooth, cylindrical, giving rise to conidia and spermatia. Conidia hyaline, smooth, ellipsoidal-fusiform to broadly cylindrical-fusiform, aseptate, apex subobtusate with irregular mucoid appendages, base broadly to acutely rounded or subtruncate. Spermatia hyaline, smooth, aseptate, variable in shape from subglobose to obovoid, produce within same conidioma as conidia.

Type species: *Pileospora piceae* J.B. Tanney.

Pileospora piceae J.B. Tanney, sp. nov. Fig. 5.6.

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Typification. Canada: New Brunswick, Northumberland County, Doaktown, 46.48035 -66.05809, isolated as endophyte from healthy surface-sterilized *Picea rubens* needle, 24 Jun 2014, J.B. Tanney NB-334-3C (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: Named after the host from which it was isolated from, *Picea rubens*.

Colonies 26–34 mm diam after 14 d in the dark at 20 C on MEA; flat, sparse aerial hyphae; margin diffuse, hyaline; surface and reverse white. Exudates and soluble

pigments absent. Mycelium consisting of hyaline, smooth, septate, branched, hyphae 1.5–3.5 μm diam.

Conidiomata on MEA pycnidial, scattered, single or clustered in small groups (2–4), submersed in agar then erumpent with maturity, dark brown to black, uniloculate, subglobose to globose, 150–500 μm diam, ostioles 80–130 μm diam. Pycnidial wall 20–45 μm wide, composed of 2–3 layers: outer layer dark, comprised of thick-walled, dark brown textura angularis; inner layer hyaline, comprised of thin-walled cells; occasionally an outermost layer present consisting of thick-walled, subhyaline to pale brown, textura angularis. Paraphyses intermingled among conidiophores, hyaline, cylindrical, aseptate, 1.5–2.5 μm diam. Conidiophores lining the inner pycnidial wall, cylindrical, hyaline, smooth, thin-walled, septate, reduced to conidiogenous cells or sparingly branched. Conidiogenous cells cylindrical, smooth, holoblastic, phialidic with prominent periclinal thickening, (11.5)14–22(–24) \times 3–4(–4.5) μm . Conidia hyaline, smooth, ellipsoidal-fusiform to broadly cylindrical-fusiform, apex subobtuse with irregular mucoid appendages up to 15 μm long, 11 μm diam (type C; Nag Raj 1993), base broadly to acutely rounded or subtruncate, aseptate, thin-walled, guttulate, appearing granular, (22–)24.5–29(–31.5) \times (8–)9–11.5(–13.5) μm , exuded in colourless droplet. Spermata formed in same conidioma as conidia, often produced in upper portion of pycnidium towards ostiole, hyaline, smooth, variable in shape, subglobose to subglobose, pyriform, ovoid, fusiform-cylindrical, aseptate, thin-walled, (4.5–)5.5–8.5(–10) \times (3–)3.5–4.5(–5) μm , exuded in colourless droplet with conidia.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Endophyte of healthy *Picea glauca* needles.

Distribution: Canada (New Brunswick)

Additional specimens and cultures examined: NB-334-1C, NB-334-3B, NB-334-3C, NB-334-4A, NB-616.

Notes: *Pileospora piceae* is morphologically similar to *Neofusicoccum* and other genera within Botryosphaerales with fusicoccum-like asexual states and irregularly-shaped spermatia. It is morphologically distinguished from such genera by its conidia, which have an irregular mucoid apical appendage. Based on the ITS and LSU phylogenies, *Pileospora piceae* is a sister to *Septorioides pini-thunbergii* and *S. strobi* (ITS P.P. = 0.98; LSU P.P. = 0.72), species forming Septorioidaceae with subcylindrical to fusiform-ellipsoidal, 1–10-septate conidia. *Pileospora piceae* is morphologically distinct from *Septorioides* by its aseptate, ellipsoidal-fusiform conidia with apical appendages. Ecologically, *Pileospora piceae* and *Septorioides* spp. share similar ecological niches as conifer needle-associates exhibiting an endophytic stage in their life history. *Septorioides* species are known only from *Pinus* needles while *Pileospora piceae* is known from *Picea mariana* and *P. rubens* needles. Septorioidaceae is a sister to the family Saccharataceae, composed largely of *Saccharata* spp., which produce both a fusicoccum-like and a pigmented diplodia-like asexual morph and are known only from Proteaceae hosts. *Pileospora piceae* pycnidia were observed on petiole bases and the

surface of a dead twig still attached to a living branch (NB-616). Funk (1965) described *Botryosphaeria piceae* from *Picea sitchensis* cankers and later reported it from *Picea glauca* (Funk 1981). An asexual state is not known for *B. piceae*, although Funk (1981) commented that the frequent association of the coelomycete *Rileyia piceae* is suggestive of its role as an asexual state or mycoparasite of *B. piceae* (Funk 1979). An isotype specimen of *B. piceae* (DAOM 105936) was re-examined for the presence of a putative asexual state, such as *Pileospora piceae*, however no such state could be found; however a connection between these two species is conceivable.

Tryblidiopsis magnesii J.B. Tanney sp. nov. Fig. 5.20

MycoBank MB XXXXXX

Typification. Canada: Ontario, Kawartha Lakes, Dunsford, 44.4834 -78.6524, ascomata on self-pruned *Picea glauca* branch, 24 Aug 2014, J.B. Tanney NB-629 (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology: Named for the Austrian mycologist Dr. Martin Magnes, who distinguished N. American *T. pinastri* subsp. *americana* from European *T. pinastri* specimens based on ascomata morphology.

Colonies 6–11 mm diam after 14 d in the dark at 20 °C on MEA; flat, sparse aerial hyphae; margin entire, hyaline; surface and reverse white to pale yellow or light orange (4A3–5A5). Exudates and soluble pigments absent. Mycelium consisting of hyaline, smooth, septate, branched, hyphae 1.5–3.5 µm diam.

Conidiomata co-occurring with ascomata on substratum, superficially similar in appearance to immature ascomata, 150–700 µm diam, globose to peg-shaped, smooth or wrinkled in appearance, semi-immersed, erumpent from bark, opening by irregular tear without ostiole, covering layer 22–40 µm thick, unilocular, locule 120–500 µm diam. Conidiophores hyaline, septate, simply branched, lining entire locular cavity. Conidiogenous cells holoblastic, sympodial, 10–15(–20) × (1.5–)2–3(–4) µm, hyaline, thin-walled, smooth, ampulliform, often swollen above midpoint before tapering to a long cylindrical collulum; conidia hyaline, filiform, falcate or sigmoid, (18–)20–26(–29) × (1–)1.5–2 µm, germination not observed on CMA, MEA, OA, or WA.

Ascomata on dead (often brittle) or senescent twigs and branches still attached to living stem of host, usually found in lower crown where self-pruning occurs. External appearance: outline circular to slightly undulate when viewed from above, pseudostipitate, black, texture leathery to pebbly, erumpent from bark, occurring singly to gregariously; young ascomata spherical to peg-shaped, becoming urceolate to discoid with maturity; 1.25–2 mm diam when mature and fresh or rehydrated, up to 1.3 mm deep, pedicel up to 350 µm tall and more evident in young ascomata. Covering stroma often opening first by slit then 3–6(–9) irregular radial fissures in humid conditions with outline appearing stellate and revealing disc-shaped, pale orange to orange grey (5A3–5B2) hymenium, subsequently retracting and covering the hymenium in dry conditions and reopening when humid conditions arise, (40–)46–75(–90) µm thick, +/- consistent thickness enveloping ascomata, sometimes thinner towards base, composed of three distinct layers: outer layer carbonaceous, *textura angularis*, 13.5–23 µm wide,

composed of (1–)2–3(–4) rows of dark brown (6F6) to black, melanised, thick-walled (1–2 μm), globose to angular cells, (4.5–)6.5–11(–12) \times (4.5–)6–9(–9.5) μm ; middle layer textura angularis, 12–26 μm wide, composed of (4–)5–6(–7) rows of hyaline, thin- to thick-walled (1 μm), globose to angular cells, (4.5–)5.5–9(–10.5) \times (4–)4.5–7.5(–9) μm , embedded in gel; inner layer textura angularis, 9–34 μm wide, composed of 2–4 rows of brown (5F8) thin- to thick-walled (1 μm), globose to angular cells, (4.5–)5–8(–8.5) \times (4–)5–6.5(–7.5) μm . Medullary excipulum well developed, 500–700 μm deep, comprised of hyaline branching hyphae embedded in gelatinous substrate, crystalline material often present, especially towards base. Subhymenium 18–30 μm thick, hyaline, textura intricata. Paraphyses exceeding length of asci, 1–2 μm wide, thin-walled, hyaline, filiform, unbranched, septate, apices rounded to clavate or occasionally ossiform, 3–4.5(–5) μm wide, frequently linked near the base by hyphal bridges. Asci arising from croziers, maturing sequentially, (120–)125–150(–165) \times (12–)13–15.5(–17) μm , cylindrical-clavate, apex obtuse to rounded, thin-walled, inamyloid, eight-spored. Ascospores biseriate, sometime uniseriate towards base, (22–)25–30(–31) \times (4.5–)5–6(–6.5) μm , ellipsoidal-fusiform to fusiform-clavate, apical end often more obtuse to clavate with basal end acute, hyaline, 0–1-septate, 2-septate ascospores rarely observed, septum median or suprmedian, covered with (1.5–)2.5–3.5(–4.5) μm thick gelatinous sheath.

Notes: *Tryblidiopsis magnesii* occurs in Eastern Canada, *T. pinastri* in Europe, and *T. sichuanensis* and *T. sinensis* in China. *Tryblidiopsis magnesii* is differentiated from *T. pinastri* by the pigmented inner layer of the covering stroma, from *T. sichuanensis* by

larger ascospores (18–24 × 4–6 µm in *T. sichuanensis*) and unilocular conidiomata, and from *T. sinensis* by the lack of hyaline apical appendages and larger, more obtuse or clavate ascospores (18–30 × 2.5–4.5 µm in *T. sinensis*). Magnes (1997) differentiated N. American specimens of *Tryblidiopsis pinastri* from their European lookalikes based on larger diam ascomata and the pigmentation of the interior stromatal covering and subsequently erected *T. pinastri* subsp. *americana*. These morphological differences, combined with rDNA phylogenetic analyses, support the recognition of the N. American subspecies as a distinct species.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Ascomata and conidiomata on self-pruned branches of *Picea glauca*, *P. mariana*, and *P. rubens*, foliar endophyte of *Picea glauca*, *P. mariana*, and *P. rubens*.

Distribution: Canada (New Brunswick, Ontario, Quebec)

Additional specimens and cultures examined: 06-381A, 06-410A, 06-415, 06-430A, 06-459A, 06-494A, NB-550, NB-630, NB-645, NB-678, NB-679, NB-680, NB-737, NB-738, NB-778.

Xylaria ellisii J.B. Tanney sp. nov. Fig. 5.26

Typification. Canada: New Brunswick, Alma, Fundy National Park, East Branch Trail, 45.6433 -65.1156, stromata on partially buried, mostly decorticate *Acer saccharum*

branch, 28 Sep 2014, *J.B. Tanney NB-623* (holotype DAOM XXXXXX, isotypes DAOM XXXXXX). Ex-type culture DAOMC XXXXXX.

Etymology. Named for the prolific mycologist Job Bicknell Ellis who, with Mordecai Cubitt Cooke, collected *Xylaria corniformis* var. *obovata* Sacc., described here as *X. ellisii*.

Colonies 32–38 mm diam after 14 d in the dark at 20 C on MEA; white, velvety, appressed, sometimes sectored; margin diffuse, hyaline; surface and reverse white. Exudates and soluble pigments absent. Mycelium consisting of hyaline, smooth, septate, branched, hyphae 1.5–3 µm diam.

Conidiophores on MEA macronematous, arising vertically from mycelium, hyaline to pale brown, smooth, cylindrical, thin-walled, dichotomously branched several times, septate, 30–60 × 3–4 µm, or occurring in synnemata, grey to olive brown (4D2–4E3), cylindrical to clavate, occurring singly, gregariously, or in clusters joined at base, up to 10 mm high by 1–3 mm diam, surface appearing powdery due to conidia. Conidiogenous cells intercalary and terminal, cylindrical, straight or undulating to geniculate, 7–16(–20) × 3–4 µm, hyaline to pale brown, producing one or more conidia holoblastically from lateral or apical regions, crater-shaped protruding secession scars 1–1.5 × 1–1.5 µm. Conidia pyriform to obovoid, subhyaline to pale brown, (5–)5.5–7(–7.5) × (2.5–)3(–3.5) µm, flattened basal scar indicating former site of attachment to conidiogenous cell.

Stromata upright, solitary, unbranched or occasionally branched once, cylindrical to spatulate or clavate, apices broadly rounded, divided into fertile head and sterile stipe, (2–)2.5–4(–5) × 0.8–1.2 cm including stipes (0.4–1.5 cm high); surface even to irregularly flattened or wrinkled, frequently cracked into a network of light brown to brownish orange (6D4–6D5) angular plates above black basal layer; stromatal interior white; stipes brownish orange to light brown (6D4–6D5) frequently with black longitudinal cracks extending from fertile head; arising from brown (767) to black pannose bases, basal mycelia often appearing iridescent. Perithecia immersed, subglobose to globose, 0.3–1 mm diam, lining the perimeter of the stromata. Ostioles conspicuous, papillate. Asci 95–130 × 6–7 μm, pars sporifera 50–80 μm, eight-spored, cylindrical, ascospores uniseriate, apical ring apparatus inverted hat-shaped, amyloid, 1.5–2 μm long. Ascospores (8–)9–9.5(–10) × (4.5–)5–5.5(–6) μm, dark brown, smooth, unicellular, ellipsoid-inequilateral, narrowly or broadly rounded ends, 1–2 guttules frequently observed, inconspicuous long, straight germ slits which are more or less the spore length, occurring on convex side, small (1.5–2 × 1.5 μm) ephemeral cellular appendage visible on less pigmented immature ascospores and disappearing as spores reach maturity.

Cardinal temperatures: Range 5–30 °C, optimum 20 °C, minimum slightly <5 °C, maximum slightly >30 °C.

Host range: Stromata on decaying *Acer saccharum* wood, foliar endophyte of *Abies balsamea*, *Picea glauca*, *P. mariana*, *P. rubens*, *Pinus strobus*, and *Vaccinium angustifolium*.

Distribution: Canada (New Brunswick, Ontario), U.S.A. (New York)

Additional specimens and cultures examined: DAOM 696463, DAOM 696464, DAOM 696466, DAOM 696480, DAOM 696488, DAOM 696489, DAOM 696492, DAOM 696493, DAOM 696503, NB-699, NB-701, NB-702, NB-703, NB-708, NB-721, NB-722, NB-723, NB-727, NB-746, CH-12, CH-15, CH-16, CH-37, CH-38, CH-4, CH-5, DT-181, DT-6, NB-236-1F, NB-236-2F, NB-236-2I, NB-285-10A, NB-285-10D, NB-285-1A, NB-285-3A, NB-285-6B, NB-285-7A, NB-285-7B, NB-285-7C, NB-285-7D, NB-365-4E, NB-365-71G, NB-365-8A, NB-366-1F, NB-366-2E, NB-366-3L, NB-366-4C, NB-382-1C, NB-382-3A, NB-382-3C, NB-382-3D, NB-382-4B, NB-391-1E, NB-391-2C, NB-391-4C, NB-406-2A, NB-406-2B, NB-406-5A, NB-421-1B, NB-437-5E, NB-464-10A, NB-487-5B, NB-487-5C, NB-487-6A, NB-487-6H, NB-488-6L, NB-505-4D, NB-746, RS9-10E, RS9-12C, T1-3B-2, T1-4B-1, T2-4A-2, T3-2A-2, T3-2B-1, T3-3A-3, T4-3A-1, T5-1A-1, T5-3B-1-1, T6-4B-1, T6-5A-1-2.

Notes: *Xylaria ellisii* is probably conspecific with *X. corniformis* var. *obovata* based on morphology, e.g.: Ju et al. (2016) recorded blackish-brown ascospores, 8–10.5 × 4.5–5.5(–6) µm from the *X. corniformis* var. *obovata* isotype. The *Xylaria corniformis* species concept is unresolved and consequently the name has been misapplied to various species within the *X. corniformis* or *X. polymorpha* aggregates (Rogers 1983). *Xylaria corniformis* s.s. is possibly a rare species known only from Sweden and Poland

collections and characterized by delicate, horn-like stromata with attenuated or sterile apices versus the robust stromata of *X. ellisii*, which also have darker coloured ascospores (Fries 1828, Læssøe 1987, Ju et al. 2009). Ju et al. (2009) concluded that *X. corniformis* var. *obovata* was an equivalent of *X. corniformis* sensu Læssøe (1987) and that this species was probably the most frequently encountered member of the *X. corniformis* complex in northern temperate regions. *Xylaria ellisii* is common on decaying fallen *Acer saccharum* branches in New Brunswick during late summer or autumn and is a frequently isolated endophyte of *Picea*, *Pinus*, and *Vaccinium angustifolium* in Eastern Canada (Richardson et al. 2014). Conspecific ITS sequences in GenBank indicate *X. ellisii* is capable of endophytically infecting a wide range of hosts.

DISCUSSION

Endophyte OTUs

Sequence similarity thresholds used for OTU delineation (e.g.: 95–97%) attempt to compensate for intraspecific sequence variation and possible sequencing errors (Jumpponen and Jones 2009, Vrålstad 2011, Lindner et al. 2013). The result can be the inflation or underestimation of species diversity based on ITS OTUs, especially when general arbitrary similarity thresholds values are applied to a phylogenetically diverse range of taxa with different levels of ITS variation. In a culture-based study using multilocus phylogenetic analyses, Gazis et al. (2011) provided a demonstration of the shortfalls of species delineation using only ITS sequences for species complexes and advocated the use of more than one gene for species delineation or, if not feasible, the

use of a higher ITS similarity threshold (99–100%) for a more accurate diversity estimate.

The number of *Picea* endophyte OTUs amplified with the increasing similarity threshold values, exhibiting a marked rise between 99% and 100% (Fig. 5.2). Increasing the similarity threshold from 98% to 99% resulted in the significant inflation of OTUs in closely-related sequences (e.g.: Rhytismataceae spp.), but did not have a significant effect on most clades, which consisted of more phylogenetically-separated OTUs. For less-studied fungi, such as Rhytismataceae spp., species concepts are primarily morphological and phylogenetic limits are poorly understood. For example, ITS variation between *Tryblidiopsis* strains ranged from 95–100%; in this case, species delineation using ITS similarity is arbitrary based on the current understanding. *Tryblidiopsis magnesii* was described based on phylogenetic data and morphological evidence available from ascomata collections; further work is needed to explore species diversity and boundaries in North American *Tryblidiopsis* collections. This highlights the importance of a well-populated phylogenetic backbone containing authenticated reference sequences for both the identification of species and cryptic species and the detection of possible artefacts (Tedersoo et al. 2010).

The inability of ITS to discriminate species complexes or distinct species sharing similar ITS sequences is established for some well-studied genera such as *Diaporthe* and *Penicillium*, leading to the use of secondary markers (Udayanga et al. 2014b, Visagie et al. 2014). In previous chapters, additional markers were used to explore the

phylogenies of species in Mollisiaceae (chapters one and two), *Diaporthe* (chapter three), and Phacidiaceae (chapter four). A major benefit of culture-based endophyte studies is the availability of viable materials facilitating further work such as multilocus phylogenetic analyses, omics studies, secondary metabolite profiling, inoculation experiments, etc. For many fungal groups, clustering of OTUs based on ITS sequences should be considered a tentative species biodiversity estimate.

***Picea* endophyte diversity**

Picea endophytes are phylogenetically diverse with the highest species diversity from Rhytismataceae, Mollisiaceae, Xylariaceae, Trichocomaceae, Dothidiaceae, Phacidiaceae, and Phaeomoniellaceae. Mollisiaceae is a large, poorly sampled family comprising at least 1000 names from *Belonopsis*, *Mollisia*, *Phialocephala*, and *Tapesia* alone. Dark septate endophyte (DSE) *Phialocephala* species are well-studied and recent evidence shows the prevalence of endophytism in *Mollisia*, a genus typically considered to be comprised of saprotrophic species (Tanney et al. 2016a). The biodiversity of Mollisiaceae *Picea* endophytes are discussed in chapters one and two and Phacidiaceae discussed in chapter three.

Xylariaceae

The family Xylariaceae (Xylariales) comprises at least 75 genera containing over 800 species (Lumbsch and Huhndorf 2011). Xylariaceae endophytes are ubiquitous in woody plant endophyte studies regardless of the host and sampling location, often exhibiting little host preference and including known saprotrophs (Dreyfuss and Petrini

1984, Petrini and Petrini 1985, Davis et al. 2003, Okane et al. 2008, U'Ren et al. 2016).

For example, *Nemania* spp. and *Annulohypoxyton multifforme* form stromata on decaying hardwood but were isolated as *Picea* endophytes in this study and reported as endophytes of other coniferous and even lichen hosts by others (Stefani and Bérubé 2006, Peršoh et al. 2010, U'Ren et al. 2012, Ellsworth et al. 2013).

Other Xylariaceae endophytes from *Picea* include *Anthostomella* spp. and *Rosellinia* sp. (Fig. 5.24). Many conifer needle and twig endophyte studies relying on morphological identification of cultures report geniculosporium- or nodulisporium-like species, which are asexual forms attributable to both *Anthostomella* and *Rosellinia* (Petrini and Carroll 1981, Sieber 1989, Laessøe and Spooner 1993, Barklund and Kowalski 1996, Hata et al. 1998). *Anthostomella* species are non-stromatic and characterized by ascomata immersed beneath a clypeus and brown ascospores with a mucilaginous sheath or hyaline dwarf cell (Lu and Hyde 2000). Kowalski (1993) reported a 28% isolation frequency for *Anthostomella formosa* as an endophyte from healthy *Pinus sylvestris* needles, with most strains isolated from first year needles. *Anthostomella conorum*, *A. pedemontana*, and unidentified *Anthostomella* spp. are also reported as endophytes of conifer needles (Petrini and Carroll 1981, Kowalski 1993, Peršoh et al. 2010, Qadri et al. 2014). *Anthostomella* species such as *A. formosa*, *A. pedemontana*, *A. rehmi*, *A. sabiniana*, and *A. sequoiae* produce ascomata on senescent or dead *Pinus* needles (Francis 1975).

Most *Rosellinia* species occur as saprotrophs but some species, such as *R. necatrix* and *R. pepo*, are destructive root rot pathogens (ten Hoopen and Krauss 2006). At least 20 *Rosellinia* species are reported from conifer trees worldwide as saprotrophs, needle endophytes, and latent pathogens (e.g.: causing needle blight) (Francis 1986, Kowalski and Andruch 2012, Farr and Rossman 2016).

Two *Picea glauca* endophyte strains representing an unknown species could not be satisfactorily placed within a Xylariales family based on ITS or LSU sequences, with ITS sequences placing them sister to *Coniocessia maxima* (Coniocessiaceae) (Fig. 5.24).

Xylaria ellisii was described to accommodate the most commonly isolated *Xylariaceae* endophyte from *Picea*. Stromata of *X. ellisii* were commonly found on decaying *Acer saccharum* branches in the same forest stands where it was isolated as a *Picea* endophyte. Endophyte ITS sequences in GenBank corresponding to *X. ellisii* originate from a variety of hosts including *Tsuga canadensis*, bryophytes (e.g.: *Hypnum* sp.), liverworts (e.g.: *Metzgeria furcata*, *Trichocolea tomentella*), and lichens (e.g.: *Flavoparmelia caperata*, *Sticta beauvoisii*, *Xanthoparmelia conspersa*) (Fig. 5.26). Corresponding *X. ellisii* stromata were commonly found in late summer and early fall only on decaying *Acer saccharum* wood, indicating a broad plant host range (e.g.: Ericaceae, Hypnaceae, Metzgeriaceae, Pinaceae, Trichocoleaceae) for the endophytic life stage and narrow host preference for the saprotrophic/stromatal life stage, although additional sampling is required to confirm the host range for the latter; Læssøe (1987), for example, examined European specimens of *Xylaria corniformis* (probably *X. ellisii*)

from *Carpinus* and *Fagus*. *Xylaria ellisii* is a common *Picea* endophyte even in conifer-dominated stands lacking *Acer saccharum* or any other hardwood hosts possibly suitable for the production of stromata, indicating *X. ellisii* is capable of persisting in the environment in the prolonged absence of a suitable primary host and suggesting a currently unknown method of transmission within the canopy. It is conceivable that the dry, powdery masses of conidia produced from conidiomata in vitro are produced on dead foliage and capable of infecting new foliage by means of air currents or insect vectors (e.g.: Pažoutová et al. 2010, Pažoutová et al. 2013). The known range of hosts that *X. ellisii* can endophytically infect includes lichens and various understory and overstory plant species with different successional statuses, allowing for its persistence across forest succession pathways and disturbances (e.g.: as an endophyte of the fire-adapted seral species *Vaccinium angustifolium*).

Richardson et al. (2014) reported the production of the antifungal compound griseofulvin by *X. ellisii* endophyte strains isolated from *Pinus strobus* and *Vaccinium angustifolium*. Griseofulvin is systemically translocated within plants, suggesting *X. ellisii* endophyte infections can increase host resistance to plant pathogens (Crowdy et al. 1956). Park et al. (2005) described griseofulvin-production in an unidentified *Xylaria* endophyte of *Abies holophylla* and showed its ability to control the development of plant diseases such as barley powdery mildew (*Blumeria graminis* f. sp. *hordei*), rice sheath blight (*Corticium sasaki*), wheat leaf rust (*Puccinia recondita*), and rice blast (*Magnaporthe grisea*). Griseofulvin and related compounds are reported from *Xylaria*

endophytes of *Asimina triloba*, *Chrysobalanus icaco*, and *Garcinia hombroniana* (Casella et al. 2013, Rukachaisirikul et al. 2013, Sica et al. 2016).

Morphologically, *X. ellisii* is referable to *X. corniformis* var. *obovata* Cooke & Ellis, *Xylaria corniformis* sensu Læssøe (1987), and *Xylaria curta* sensu Rogers (1983). The *Xylaria corniformis* aggregate is a polyphyletic morphotaxonomic concept comprising species characterized by stromata with a wrinkled surface and a thin outer layer covering that gradually cracks into fine scales with maturation, and includes *X. bipindensis*, *X. cuneata*, *X. curta*, *X. divisa*, *X. feejeensis*, *X. humosa*, *X. luteostromata*, *X. maumeei*, *X. montagnei*, *X. plebeja*, *X. ramosa*, *X. rhytidophloea* (Ju et al. 2009, Hsieh et al. 2010, Ju et al. 2016). Rogers (1983) noted the taxonomic confusing surrounding *X. corniformis* and its misapplication to *X. bulbosa*, *X. castorea*, *X. curta*, and other morphologically similar species, recommending that *Xylaria* taxonomy would be best served if the name *X. corniformis* were no longer used. Ju et al. (2009) noted that *X. corniformis* var. *obovata* was probably the most frequently encountered member of the *X. corniformis* complex in northern temperate regions and considered *X. corniformis* and *X. corniformis* var. *obovata* as distinct species, but refrained from making a formal taxonomic decision pending additional evidence. Based on the *RPB2* phylogeny, *X. ellisii* is weakly supported (P.P. = 0.63) sister to *X. laevis* and other species within the *X. cubensis* aggregate, however additional *RPB2* sequences for related *Xylaria* species are needed to generate a more comprehensive phylogeny (Fig. 5.27). Several herbarium specimens identified as *Xylaria corniformis* from *Acer* sp. wood in Ontario and Quebec were morphologically similar to *X. laevis* and resulting ITS sequences showed they

formed a clade sister to *X. longipes* and *X. primorskensis* and were distinct from *X. ellisii* (Fig. 5.25). *Xylaria ellisii* was described here because of morphological differences between *X. corniformis* s.s. and *X. ellisii*, the necessity to name this ubiquitous endophyte and a saprotroph in Eastern Canada, and the need to delineate boundaries in species complexes with good species concepts connected to authenticated reference sequences and specimens.

Xylariaceae endophytes are common but often difficult to identify to species rank because of a lack of reference sequences and limited taxonomic resolution of asexual states based on current knowledge; however, studies have provided connections between Xylariaceae stromata and corresponding endophytes (Okane et al. 2008, Thomas et al. 2016, U'Ren et al. 2016). Endophyte studies should therefore incorporate broad field surveys to identify and sequence stromata from the host environment in an attempt to identify unknown Xylariaceae endophytes.

Trichocomaceae

Trichocomaceae (Eurotiales) species are predominantly saprotrophs which are ubiquitous in the environment. Thus improper surface sterilization of plant materials may deceptively reveal “endophytic” Trichocomaceae species. Doubtful endophytic *Penicillium* species isolated in this study include *P. chrysogenum*, *P. lanosum*, and *P. miczynskii*, although *P. lanosum* is reported as a *Sorbus* endophyte (Frisvad et al. 2006) while *P. miczynskii* is reported as an epiphyte (Lorenzi et al. 2006a, Osono 2007). Endophytic *Penicillium* species are convincingly reported in some studies and it is likely

that some species are functional components of niche ecosystems and not simply incidental contaminants (Peterson et al. 2003, Peterson et al. 2005, Vega et al. 2006, Visagie et al. 2016).

Penicillium sect. *Thysanophora* comprises conifer-specific species exhibiting highly divergent morphologies compared to related *Penicillium* s.s. species, notably dark colonies, thick melanised conidiophores, and secondary growth of the stipe by the proliferation of an apical penicillius (Houbraken and Samson 2011), characters that resulted in its original placement in *Haplographium* and then its own genus *Thysanophora* (Kendrick 1961a). *Penicillium* sect. *Thysanophora* species are mostly known from conifer needle litter but are reported as *Picea* endophytes in other studies (Müller and Hallaksela 1998, Stefani and Bérubé 2006, Koukol et al. 2012). Eight accepted species comprise sect. *Thysanophora*, however many more undescribed species are likely to exist; based on the *BenA* phylogeny presented in this study, the endophytic *Picea* strains from New Brunswick represent three distinct novel species and additional strains isolated from fallen *Picea* needles in the litter layer represent a fourth novel species (Fig. 5.15). Sequences of strains isolated from additional conifer or environmental samples included in the *BenA* phylogeny indicate more undescribed species and the application of *Penicillium glaucoalbidum* (as *Thysanophora penicillioides*) for many distinct species. *Penicillium* sect. *Thysanophora* conidiophores and sclerotia erumpent from stomata were frequently observed on dead and senescent *Picea* needles still attached to living branches or in the litter layer, or from fresh needles incubated in damp chambers (Fig. 5.14).

Penicillium glaucoalbidum is reported to cause significant weight loss and reduction in the phenolic compound p-hydroxyacetophenone in cast *Picea* needles (Koukol et al. 2008), suggesting that species are latent saprotrophs. Conifer needles are a recalcitrant substrate and probably exert a strong selective effect on potential fungal decomposers, driving some degree of host specificity or preference (Kendrick 1962). Mechanisms driving the plant-association and host-specificity of *Penicillium* sect. *Thysanophora* have not been thoroughly investigated, although Black and Dix (1976) reported that high concentrations (up to 600 µg) of ferulic acid, a phenolic compound that is a building block of lignocelluloses including lignin, significantly stimulated conidia germination and germ hyphal length in *P. glaucoalbidum* while inhibiting that of common soil fungi (e.g.: *Cladosporium herbarum*, *Penicillium spinulosum*, *Trichoderma viride*). Species in sect. *Thysanophora* share morphological, genetic, and ecological apomorphies distinct from related sections and warrant further investigation into their biodiversity and host interactions. Furthermore, this clade suggests that other *Penicillium* species may have more specific ecological niches in nature than currently realized because of a traditional focus on air-, food- and soil-borne isolates.

Rhytismataceae

Rhytismataceae (Rhytismatales) contains 55 genera and ca. 730 species of endophytic, parasitic, and saprotrophic plant associates, many of which are exclusively associated with conifers (Kirk et al. 2008, Lantz et al. 2011). The general ascomata morphology of Rhytismataceae consists of an ascoma immersed in host tissue with a

pigmented shield-like clypeus (Fig. 5.21), which is closed in dry conditions and open in humid conditions to reveal a hymenium consisting of paraphyses that are simple or branched, filiform, free or embedded in a gelatinous epithecium, often connected by hyphal bridges near the bases, with cylindrical to clavate or circinate apices, and asci with undifferentiated thin-walled inamyloid apices, which forcibly eject (4–)8 ascospores that are typically filiform and surrounded in gelatinous sheaths (Darker 1967, Sherwood 1980, Lantz et al. 2011). *Cudonia* and *Spathularia* (Cudoniaceae, Rhytismatales) form a morphologically and ecologically divergent clade, producing relatively large (up to 10 cm tall) clavate to fan-shaped, capitate ascomata with sterile stipes and fertile apical regions. Despite these gross morphological differences, paraphyses, asci, and ascospores of *Cudonia* and *Spathularia* are typical of Rhytismataceae and the veil that covers the developing hymenium may be homologous to the covering layer of some Rhytismataceae species (Ge et al. 2014). Molecular phylogenetic work shows Cudoniaceae is nested within, and therefore synonymous with, Rhytismataceae (Lantz et al. 2011, Ge et al. 2014). This clade probably represents the transition from an endophytic or pathogenic life strategy to that of a saprotroph within Rhytismataceae.

The genus *Lophodermium* (Rhytismataceae) comprises over 100 species predominately described from conifers (Kirk et al. 2008). Larkin et al. (2012) reported that Rhytismataceae fungi comprised 50% of *Pinus monticola* endophytes, with 93% of these Rhytismataceae endophytes belonging to *Lophodermium* s.l. *Lophodermium* is polyphyletic; the type species *L. arundinaceum* belongs to a strongly supported clade of mostly grass-associated species while other *Lophodermium* species occur throughout

Rhytismataceae (Lantz et al. 2011). In this study, *Lophodermium cf. piceae* was the most abundant endophyte isolated from *Picea* (Fig. 5.18). *Lophodermium piceae* was previously assumed to be associated with both European and North American *Picea* needles; however, cultural studies using ITS rDNA sequences have indicated the presence of cryptic species, including distinct North American species (Stefani and Bérubé 2006). *Lophodermium cf. piceae* strains in this study are readily distinguished from European strains using ITS barcodes, further supporting the distinction of North American *Lophodermium cf. piceae* from *L. piceae* s.s. (Fig. 5.17). The 99% similarity threshold resulted in five *Lophodermium cf. piceae* clusters that were not apparently host-specific, although future work should include additional unlinked loci to test the distinction of these clusters under the principles of the genealogical concordance phylogenetic species recognition concept (GCPSR; Taylor et al. 2000) and improved sampling involving comparable number of *Picea glauca*, *P. mariana*, and *P. rubens* isolates for better comparison of host specificity. Stefani and Bérubé (2006) identified five North American *L. cf. piceae* subgroups based on ITS sequences; subgroup 1 was closely related to European *L. piceae*, subgroups 2 and 4 were specific to *Picea glauca*, subgroup 3 was specific to *Picea mariana*, and subgroup 5 was found on both *P. glauca* and *P. mariana*. Recent molecular studies have resulted in the identification and description of cryptic *Lophodermium* species previously undetected and misidentified because of morphological similarities with related species (Sokolski et al. 2004, Hou et al. 2009, Gao et al. 2013, Koukol et al. 2015, Oono et al. 2015). The description of novel

phylogenetic species will assist in the recognition of host and biogeography specificity or preference in cryptic *Lophodermium* species of North American conifers.

Another taxonomic issue surrounding *Lophodermium piceae* s.s. and *L. cf. piceae* is that they form a basal clade with *Lirula macrospora*, distantly related from *Lophodermium arundinaceum* and other well-known *Lophodermium* species associated with conifer needles including *L. conigenum*, *L. nitens*, and *L. pinastri* (Lantz et al. 2011). Addressing the taxonomy of *Lophodermium* is difficult based on current data; however monophyletic generic concepts must be pursued. Accepting a narrow *Lophodermium* s.s. concept will restrict the genus to mostly grass-associated species, resulting in extensive taxonomic changes for important conifer-related species occurring throughout Rhytismataceae. Alternatively, *Lophodermium* could be conserved with a new type to reduce the number of name changes and encompass species that are economically important (e.g.: pathogens) and in more frequent use than those in the *L. arundinaceum* clade. For example, *L. pinastri* could be selected as the new type species for *Lophodermium* (e.g.: Gams et al. 2005). In their guidelines for introducing new fungal genera, Vellinga et al. (2015) recommended that all recognized genera be monophyletic, not only those that are the focus of a particular study. This presents a challenge for large, undersampled polyphyletic genera such as *Lophodermium*, which effectively spans the family Rhytismataceae. A piecemeal approach to solving taxonomic issues will undoubtedly result in the description of novel but possibly questionable genera, but insufficient data exists to make strongly supported inferences about monophyly throughout the lineage. Efforts to sequence additional protein-coding

loci (e.g.: *TEF1- α* , *RPB2*) for new collections must be made to provide material for a future taxonomic treatment of Rhytismatales.

The basal position of *L. piceae* means it can be convincingly placed within a novel genus to accommodate its phylogenetic distinctiveness without taxonomic disruption to other Rhytismataceae taxa. However, based on rDNA phylogenies, *Lophodermium piceae* is sister to *Lirula macrospora* (Lantz et al. 2011, Fan et al. 2012, Wang et al. 2014b). *Lirula* comprises 11 currently accepted species known from *Abies* and *Picea* needles. Darker (1967) erected *Lirula* to accommodate related nervisequious species previously placed in *Hypoderma* or *Lophodermium*. *Lirula* species are characterized by hypophyllous, nervisequious, mostly linear, black to brown ascomata that often extend along the entire needle length and develop on needles at least two years of age. Epiphyllous pycnidia, which are more or less continuous along the sulcus or in two parallel rows on each “wing” of the needle, are formed on one-year-old needles. Ascospore shape distinguishes *Lirula* (clavate to clavate-cylindrical) from other nervisequious genera such as *Isthmiella* (bifusiform) and *Virgella* (bacillar) (Darker 1967, Funk 1985). Some *Lirula* species are implicated in needle blight and needle cast of *Abies* and *Picea* (Ziller 1969, Scharpf 1988, Kaneko 2003, Horst 2013). A combined ITS and LSU phylogeny by Fan et al. (2012) showed *Lirula* is polyphyletic by the inclusion of *L. piceae* (although weakly supported; bootstrap value = 53, P.P. = 0.76) and the distantly-related species *Lirula exigua*, which differs morphologically from other *Lirula* species by its scattered pycnidia, amphigenous ascomata containing paraphyses with recurved tips, and relatively small ascospores (Kaneko 2003).

Should *Lophodermium piceae* be transferred to *Lirula*? The phylogenetic placement of *Lophodermium piceae* within *Lirula*, a genus comprising a more-or-less coherent morphotaxonomic concept, is unexpected because *Lophodermium piceae* ascomata are amphigenous, 0.60–1.9 × 0.5–0.77 mm, and bear filiform ascospores. In the most comprehensive Rhytismatales phylogeny to date, Lantz et al. (2011) only included partial 18S nuc rDNA gene (SSU) region sequences of *Lirula macrospora* (HM143807) which, when compared to *Lophodermium piceae* (HM143825), do not suggest both species are congeneric because of sequence dissimilarity for this highly conserved rDNA region, e.g.: identities = 805/863 (93%), gaps = 33/868 (3%). A *Lirula macrospora* sequence referenced in several studies (LSU: HQ902152, ITS: HQ902159) originating from *Picea abies* in Germany is closely related to the *Lophodermium piceae* LSU sequence used by Lantz et al. (2011), e.g.: HM140551; identities = 835/861 (97%), gaps = 1/861 (3%) but distantly related to other *L. piceae* based on ITS sequences, e.g.: AY775681; identities = 381/425 (90%), gaps = 10/424 (2%) (Fan et al. 2012, Lei et al. 2013, Wang et al. 2013b, Wang et al. 2014b, Zhang et al. 2015). *Lirula* is represented by few sequences and the type species, *Lirula nervisequa*, is absent in GenBank; furthermore, there are misidentified *L. macrospora* sequences in GenBank (e.g.: AF203472 = *Tryblidiopsis* cf. *pinastri*, AF462440 and AF462441 are sister to *Therrya*) (Wang et al. 2013b). *Lophodermium piceae* is clearly not congeneric with *Lophodermium* s.s. and phylogenetic evidence based on currently available sequence data suggests it is sister to, but not congeneric with, *Lirula*. Future research should include the description of a novel genus to accommodate *Lophodermium piceae* and the

formal distinction of European and North American *L. piceae* species, a polyphasic investigation of the variation in N. American “*L. piceae*” strains, and the epitypification and sequencing of *Lirula macrospora* and *L. nervisequa* from type locales.

Coccomyces is a large genus of at least 120 recognized species occurring globally and associated with plants in 61 families, especially recently dead plant material of Pinaceae, Ericaceae, Fagaceae, and Lauraceae hosts (Sherwood 1980, Wang et al. 2013a). The ecology and life-history of *Coccomyces* species is largely unknown and any generalizations must take into account the polyphyly of *Coccomyces* s.l. (Lantz et al. 2011). However, species of *Coccomyces* that sporulate only on bark or recently killed twigs have been detected as foliar endophytes (Stone et al. 2004). Hirose et al. (2013) reported evidence revealing *Coccomyces sinensis* latently infects healthy leaves as an endophyte and persists after senescence and abscission as a saprotroph, where it then produces ascomata from the host substrate. In this study, *Coccomyces* strains isolated as foliar endophytes were connected with species that sporulate on bark of living *Picea* trees (*C. irretitus*; Fig. 5.19) or recently killed or self-pruned *Pinus strobus* branches (*C. strobi*; Fig. 5.22). *Coccomyces* species are numerous but poorly sampled and underrepresented by reference sequences; consequently, many *Picea* endophytes placed in *Coccomyces* s.l. based on ITS and LSU sequences were unidentifiable. Therefore identification and sequencing of endophyte host-associated *Coccomyces* ascomata from various aboveground senescent tissues will invariably result in the subsequent identification of common but currently unknown Rhytismataceae endophytes.

The genus *Colpoma* is polyphyletic and comprises about 24 species (Index Fungorum 2016a) characterized by large and deeply embedded ascomata, which are morphologically similar to *Coccomyces* but open longitudinally versus radially (Johnston 1991, Lantz et al. 2011). Unidentified Rhytismataceae *Picea mariana* endophytes (NB-221-5A and 08-052B) were connected with *Colpoma* cf. *crispum* ascomata collected from wood and bark of a fallen *Picea mariana* branch (Fig. 5.21). This species is phylogenetically distinct from other *Colpoma* species and sister to *Pseudographis pinicola*, a morphologically unique Rhytismataceae species that is considered rare and known from *Picea* and *Pinus* bark (Dennis 1968). *Pseudographis pinicola* is characterized by a waxy hymenium and amyloid ascospores with up to seven septa. The only known sequence of *P. pinicola* was derived from an original collection of ascomata on the bark of a living *Larix laricina* tree in New Brunswick.

Tryblidiopsis is a small genus comprising four species, five including *T. magnesii*, which occur on dead conifer twigs and branches (Index Fungorum 2016a). *Tryblidiopsis pinastri* is specific to *Picea* and occurs as a cambium endophyte, producing ascomata on recently dead branches and thought to occur in North America (Livsey and Minter 1994, Barklund and Kowalski 1996). *Tryblidiopsis* strains were commonly isolated from *Picea* needles in this study, including ITS sequences that were distinct from European *Tryblidiopsis pinastri* and comprising eight clusters based on a 99% similarity threshold (Fig. 5.1). *Tryblidiopsis pinastri* was thought to have a circumpolar boreal distribution with *Picea* species, however Wang et al. (2014a) identified three cryptic *Tryblidiopsis* species from *Picea* spp. in China, describing *T. sichuanensis* and *T. sinensis* but leaving

the third species undescribed because of the immaturity of ascomata specimens. Magnes (1997) observed that North American *T. pinastri* had larger ascomata and the interior stomatal covering was conspicuously pigmented, subsequently erecting *T. pinastri* subsp. *americana* to distinguish the North American and European morphotypes. In this study, a number of *Tryblidiopsis* foliar endophyte strains from *Picea glauca* and *Picea rubens* were connected with ascomata collected from self-pruned *Picea glauca* branches based on identical ITS and LSU sequences (Fig. 5.17). Ascomata exhibited the pigmented stomatal covering described by Magnes (1997) and resulting ITS and LSU sequences were not conspecific with other *Tryblidiopsis* species known from *Picea*; therefore, this species was described as *Tryblidiopsis magnesii* sp. nov. (Fig. 5.20). These findings suggest the presence of additional cryptic *Tryblidiopsis* species of North American spruce and indicates *Tryblidiopsis* spp. are capable of persisting as both foliar and cambial endophytes. Strains resolving into four OTUs in two distinct clades were sister to *Tryblidiopsis* s.s. and could not be placed in genera based on currently available reference sequences (Figs. 5.1, 5.16); their actual identities can only be speculated upon and necessitate the further collection of Rhytismataceae ascomata from *Picea* trees.

Dothidiaceae

Dothidiaceae (Dothideales) endophytes of *Picea* isolated in this study included strains related to *Phaeocryptopus*, *Rhizosphaera*, and *Sydowia*. Overall, Dothidiaceae comprises many species known from needles, bark, and branches of conifer trees and

other woody plant hosts. For example, *Atramixtia arboricola* described from black subicula on twigs of declining *Picea glauca* in Alberta, Canada (Tsuneda et al. 2011). Dothidiaceae genera include species of so-called black meristematic fungi that are frequent biotrophic, necrotrophic, or saprotrophic plant associates (Tsuneda et al. 2011, Thambugala et al. 2014). Colonies are often slow growing, dematiaceous, and yeast-like, exhibiting pleomorphic conidiogenesis including endoconidia (Tsuneda et al. 2001, Tsuneda et al. 2011). The general ecology of Dothidiaceae species associated with conifer trees is not well understood, with evidence of species occupying several niches, e.g.: *Scleroconidioma sphagnicola* is a reported necrotrophic parasite on moss, a saprotrophic colonizer of wood, and an endophyte and early colonizer of conifer needle litter, (Tsuneda et al. 2000, Koukol et al. 2006, Koukol et al. 2012, Haňáčková et al. 2015).

Rhizosphaera encompasses seven species, which are generally accepted as asexual morphs of *Phaeocryptopus*, although this relationship has never been conclusively established (Winton et al. 2007, Kirk et al. 2008). All species are described from conifer needles and produce erumpent conidiomata from stomata, with the waxy stomatal plug frequently retained on the conidioma apex (Supplementary Figure S1; Funk 1985). Conidiomata are simple, consisting of a single-celled wall or an amorphous coat, bearing ovate to elliptical conidia produced enteroblastically from the walls of conidiogenous cells (Butin and Kehr 2000). A hormonema-like synanamorph is usually observed in *Rhizosphaera* cultures (Butin and Kehr 2000). *Rhizosphaera kalkhoffii* and *Phaeocryptopus nudus* were common endophytes of healthy *Picea* needles and ITS and

LSU sequence suggest the presence of two putatively novel species (Fig. 5.9).

Rhizosphaera is a reported endophyte that is probably restricted to needles (Ganley et al. 2004, Ganley and Newcombe 2006, Yuan et al. 2011); however, there are also scant reports of *R. kalkhoffii* from twigs (Shriner and Grand 1974, Sieber 1989) and Diamandis (1978) isolated *R. kalkhoffii* from pulvini following the death of affected needles and reported the fungus viable for almost a year after needles were shed. *Rhizosphaera kalkhoffii* often co-occurs with *Lophodermium piceae* on *Picea abies* and its conidiomata are abundant on fallen needles (Livsey and Barklund 1992), although Scattolin and Montecchio (2009) showed that the abundance of *R. kalkhoffii* was negatively correlated with the abundance of *L. piceae*. *Rhizosphaera kalkhoffii* is implicated in needle cast and blight in *Abies* and *Picea* and is also reported from asymptomatic foliage (Kobayashi 1967, Juzwik 1993, Butin 1995, Manter and Livingston 1996, Stone et al. 2004, Talgoslash and Stensv 2012).

Additional work is required to clarify the taxonomy of *Rhizosphaera* and determine its relationship with *Phaeocryptopus* s.s. Type sequences or ex-type strains do not exist for the type species of *Rhizosphaera*, *Rhizosphaera abietis*, or *Phaeocryptopus*, *P. nudus*, as well as *Rhizosphaera kalkhoffii* and *R. pini*. Efforts to epitypify these species under the guidelines of Ariyawansa et al. (2014) will assist in the delineation of *Rhizosphaera* from related genera, provide evidence for or against the synonymy of *Phaeocryptopus* with *Rhizosphaera*, and facilitate rapid and confident species identification of strains and better understand pathogenicity of species.

Celosporium laricicola and a putative undescribed *Celosporium* sp. [compared to *C. laricicola* ITS sequence FJ997267: identities = 501/509 (98%); gaps = 3/509 (0%)] were isolated as *Picea* endophytes. *Celosporium* is a monotypic genus consisting of *C. laricicola*, a black meristematic fungus described from subicula of declining *Larix lyallii* branches in Alberta, Canada (Tsuneda et al. 2010). Based on a combined ITS, SSU, and LSU phylogeny, Thambugala et al. (2014) considered *Celosporium laricicola* incertae sedis in Dothideales, sister to Dothideaceae and Aureobasidiaceae; the ITS phylogeny strongly supports (P.P. = 0.97) *C. laricicola* as a sister to Dothideales.

Sydowia polyspora was isolated as a *Picea* endophyte in this study and reported as an endophyte in other conifers (Petrini et al. 1989, Kowalski 1993, Linaldeddu et al. 2003, Gil et al. 2009, Martin et al. 2012, Bullington and Larkin 2015). It is also known as a saprotroph on dead foliage, branches, stems, and cones of a wide variety of conifer hosts and is a common isolate from surface-sterilized *Abies* seeds (Smerlis 1970, Minter 1981, Talgø et al. 2010a). *Sydowia polyspora* is a common saprotroph or weak facultative parasite but is also known to occasionally cause serious damage to conifers, with inoculation studies establishing its pathogenicity in *Abies*, *Larix*, *Pinus*, *Thuja*, and *Tsuga* species (Smerlis 1970, Talgø et al. 2010b, Tinivella et al. 2016). Disease symptoms include prematurely chlorotic needle bands or spots, cast needles, cankers, and branch or leader death (Batko et al. 1958, Smerlis 1970, Talgø et al. 2010b). Ridout and Newcombe (2015) reported a 4.7% increase in *Dothistroma* needle blight disease symptoms when *Dothistroma septosporum* was co-inoculated with *Sydowia polyspora* in *Pinus ponderosa* trees. However, *Sydowia polyspora* is probably a species complex

requiring DNA barcodes to delineate morphologically indistinguishable species, an important consideration when reviewing the literature citing "*S. polyspora*" on conifers based on morphological identification, especially its *Hormonema* asexual state (Hambleton et al. 2003, Bills et al. 2004).

Phaeomoniellaceae

The monotypic order Phaeomoniellales, sister to Chaetothyriales and Verrucariales, was described to accommodate the genera *Celothelium*, *Dolabra*, *Moristroma*, *Phaeomoniella*, and *Xenocylindrosporium* (Chen et al. 2015). Crous et al. (2015d) recently erected six novel genera based primarily on rDNA phylogenies for novel and known Phaeomoniellaceae species previously placed in *Phaeomoniella*. Most Phaeomoniellaceae species are plant-associates known as endophytes, saprotrophs, and pathogens; for example, *Phaeomoniella chlamydospora* is associated with Petri disease and esca in *Vitis vinifera* (Crous and Gams 2006, Damm et al. 2010). Phaeomoniellaceae species are also reported as epiphytes and twig and needle endophytes of conifers (Lee et al. 2006, Alonso et al. 2011, Botella and Diez 2011, Broders et al. 2015, Sanz-Ros et al. 2015). In this study, *Picea* strains representing five unique undescribed Phaeomoniellaceae species are reported (Fig. 5.16). *Phaeomoniella chlamydospora* is well-studied because of its association with disease in grapevines, however the ecology of Phaeomoniellaceae species associated with conifer trees is unknown and warrants further investigation.

Cenangiaceae

Pärtel et al. (2016) recently provided a comprehensive treatment of Cenangiaceae and related families incorporating multilocus phylogenetic analyses and morphological studies. Cenangiaceae comprises the former family Hemiphacidiaceae, including species associated with conifer foliage or twigs such as *Cenangium ferruginosum*, *C. japonicum*, *Crumenulopsis sororia*, *Fabrella tsugae*, *Heyderia abietis*, *H. puisilla*, *Rhabdocline* spp., *Sarchotrochila longisporum*, *S. macrospora*, and *Vestigium trifidum*. Pärtel et al. (2016) suggested *Hysterostegiella* belonged to Cenangiaceae based on morphology but lacked molecular evidence; the collection of *H. typhae* (NB-638) suggests it belongs to this family (Fig. 5.8). In this study, *Heyderia abietis* was collected on *Picea rubens* needles in the litter layer but was not detected as an endophyte. *Heyderia* and *Chlorencoelia* produce relatively large, well-developed ascomata and are probably saprotrophs, while other Cenangiaceae species produce highly reduced ascomata and are associated with living plant tissues at some point during their life history.

Ascomata of a *Cenangium* sp. found on self-pruned *Pinus strobus* twigs were connected with unidentified *P. strobus* endophytes using ITS and LSU sequences. Before the collection and sequencing of this specimen (NB-663), these endophytes were previously unidentifiable to the generic rank. *Cenangium* sp. is similar to *Cenangium ferruginosum*, the type species of *Cenangium*, and *C. japonicum*; all species possess inamyloid, asymmetrical, subconical ascus apices, broadly ellipsoidal-ovoid ascospores

with gelatinous sheaths, and are found on dead *Pinus* twigs (Pärtel et al. 2016).

Cenangium sp. is highly supported (P.P. = 1) sister to a clade containing *C. ferruginosum* and *C. japonicum* (Fig. 5.8); *Cenangium ferruginosum* and *C. japonicum* exhibit a yellowish to reddish-brown ionomidotic reaction and ascospores stain a rose colour in KOH (K. Partel pers. comm.), reactions not observed in *Cenangium* sp. (NB-663).

Examination of additional specimens identified as *C. ferruginosum* (DAOM 23237, 109176, 128467) or *C. abietis* (= *C. ferruginosum*; DAOM 12653, 142653) from *P. strobus* in Ontario, Canada shows that they are identical to NB-663, suggesting that this is a common species that is likely undescribed and was misidentified by previous mycologists. Based on the ITS phylogeny, endophytes isolated from conifers (*Juniperus scopulorum* and *Pinus taeda*), *Symphyogyna* sp., and *Flavoparmelia praesignis* are conspecific with *Cenangium* sp. (NB-663), e.g.: *Pinus taeda* endophyte KM519238; identities = 493/497 (99%), gaps = 0/497 (0%). These endophyte sequences were accessioned into GenBank as “Leotiomyces sp.” or “Fungal sp.” because of a lack of related reference sequences. One of the most common endophyte OTUs detected in *Pinus taeda* (“caw049”), represented by 41.7% of the total ITS clones, was an unidentified Leotiomyces sp. and is actually *Cenangium* sp. (NB-663) (Oono et al. 2015).

Cenangium ferruginosum is commonly isolated as an endophyte of *Pinus* needles, buds, and twigs with reports of apothecia restricted to dead *Pinus* twigs and branches (Helander et al. 1994, Jurc et al. 2000, Alonso et al. 2011). Smerlis (1973) confirmed the reported pathogenicity of *C. ferruginosum* by inoculation tests using a

strain originating from *Pinus sylvestris*, where it caused small cankers in *Picea glauca*, *P. mariana*, *Pinus contorta*, *P. resinosa*, *P. sylvestris*, and *P. strobus*. *Cenangium ferruginosum* is probably a weak opportunistic pathogen, with reports of *Cenangium* dieback in *Pinus* spp. from Europe and Japan usually associated with several other pre-existing biotic or abiotic agents predisposing the host to further damage by opportunistic pathogens (Kessler 1993, Butin 1995, Duda and Sierota 1997, Koiwa et al. 1997, Kowalski 1998, Kunca and Leontovyc 2013). Furthermore, necrosis lengths measured in healthy *Pinus halapensis* seedlings inoculated with *C. ferruginosum* were not significantly different than those from the control wounding treatment; *C. ferruginosum* was re-isolated from 89% of the inoculated seedlings (Santamaria et al. 2007). Jurc and Jurc (1997) hypothesised that the ubiquity of *C. ferruginosum* foliar endophytic infections enabled the fungus to invade twigs of drought-stressed *Pinus nigra* trees and that its constant presence across the landscape explained why epidemics could occur rapidly over large areas during periods of stress. Helander et al. (1994) suggested that *C. ferruginosum* systemically infect *Pinus* needles by starting from the twigs and moving through the petioles in the needle base, or vice versa. In North America, *Cenangium ferruginosum* is mostly considered a saprotroph or weak parasite of suppressed or weakened lower branches (Scharpf 1993, Harrison 2009), although there are scant reports of moderate-to-severe *Cenangium* dieback in *Pinus resinosa* and *P. sylvestris* plantations (Foster 1976, Weir and Lawrence 1976, Applejohn et al. 1980) and *C. abietis* (= *C. ferruginosum*) was the reported causal agent of a needle blight on lower branches of *Pinus strobus* after a prolonged drought (Fink 1911).

The misidentification of *Cenangium* sp. (NB-663) as *C. ferruginosum* shows the need for further taxonomic work to determine the species diversity of *Cenangium* within North America. It is possible that the morphological variation and varying reports of pathogenicity, for example in *Pinus* subs. *Pinus* hosts (*Pinus halapensis*, *nigra*, *P. resinosa*, *P. sylvestris*) but rarely *P. strobus*, are a result of distinct species that have gone unnoticed.

Notes on other selected taxa

Septorioideaceae (Botryosphaerales) is a monotypic family recently described to accommodate a genus sister to Saccharataceae (Broders et al. 2015). *Septorioides* consists of two species, *S. pini-thunbergii* and *S. strobi*, both described from symptomatic *Pinus* needles. The ecology of *Septorioides* species is largely unknown; however, evidence suggests they are probably weakly pathogenic latent endophytes (Kaneko et al. 1989, Kihara et al. 2015, Wyka and Broders 2016). *Pileospora piceae* sp. nov., a *Picea rubens* endophyte, is basal to *Septorioides* based on ITS and LSU phylogenies (Figs. 5.21, 5.22) and is morphologically distinct, bearing more resemblance to *Neofusicoccum* than *Septorioides*. GenBank accessioned sequences from other endophyte studies reveal that this species was isolated from *Picea mariana* in Eastern Quebec (Higgins et al. 2007). The biology of *Pileospora piceae* is unknown, however it exists as a foliar endophyte and pycnidia were observed on the petiole bases and surface of a dead *Picea rubens* twig still attached to a living branch.

Hyphodiscus sp. (Hyaloscyphaceae) was initially unidentifiable to genus based on cultural morphology and rDNA sequences, with the closest related ITS sequence being an unidentified Helotiales sp. *Pinus monticola* endophyte [HQ845747; identities = 458/489 (94%), gaps = 4/489 (0%)]. The identity of this endophyte was revealed following its connection with small (0.3–0.4 mm diam) pale to white apothecia occurring on the surface of living *Picea mariana* and *P. rubens* twigs and reaching maturity in mid-summer (Figs. 5.10, 5.11). This species is undescribed and, based on ITS and LSU sequences, is sister to a *Hyphodiscus* clade comprising species known for their fungicolous habitat, such as *Hyphodiscus hymeniophilus* and *Phialophora brevicollaris* (Stip Helleman pers. comm.). Future collections will be made to complete the description of this fungus for publication.

Phaeocryptopus gaeumannii is a well-studied pathogen that causes Swiss needle cast of *Pseudotsuga menziesii*, which can reduce tree volume growth by up to 23–50% (Maguire et al. 2002, Manter et al. 2003, Ritóková et al. 2016). Despite its current generic designation in *Phaeocryptopus*, *P. gaeumannii* is phylogenetically unrelated to *Phaeocryptopus* s.s. (Dothideaceae) and is actually in the Mycosphaerellaceae (Capnodiales) (Winton et al. 2007). In this study, a putative undescribed Mycosphaerellaceae sp. isolated as a *Picea rubens* endophyte is sister to *Phaeocryptopus gaeumannii* (Fig. 5.12). Two GenBank sequences of *Picea mariana* endophytes from Quebec are conspecific with Mycosphaerellaceae sp. Strains misidentified as *Conoplea elegantula* that are actually conspecific with Mycosphaerellaceae sp. produced six new and two known isocoumarins and related

secondary metabolites, three of which were toxic to spruce budworm cells (Findlay et al. 1995). Immature Mycosphaerellaceae-like perithecia were commonly observed on living *Picea* twigs, however few mature specimens were observed and cultures could not be obtained (Fig. 5.13). Efforts to collect and culture mature specimens are needed to determine if these perithecia represent the endophytic Mycosphaerellaceae sp. Furthermore, taxonomic revision of *Phaeocryptopus gaeumannii* is needed as this species is unrelated to *Phaeocryptopus* s.s., rendering the genus polyphyletic. If *Phaeocryptopus nudus* is congeneric with *Rhizosphaera*, the name *Phaeocryptopus* should probably be applied to *P. gaeumannii* because of its economic importance and frequent usage among foresters and forest pathologists. *Rhizosphaera* (1907) has priority over *Phaeocryptopus* and should be used for the remaining Dothideaceae *Phaeocryptopus* spp. as *Rhizosphaera* s.s.

Strains of four OTUs related to *Pseudoplectania melaena* and *P. nigrella* were isolated from *Picea rubens*. *Pseudoplectania* (Sarcosomataceae, Pezizales) comprises 12 species, with *P. melaena* and *P. nigrella* known for their large (up to 3 cm diam) conspicuous, black cup-shaped apothecia found in spring on decaying *Abies* and *Picea* branches and logs or litter, respectively (Dennis 1968, Farr and Rossman 2016). Some consider *Pseudoplectania melaena* an indicator species of natural to virgin forests, although this may simply be a reflection of its preferred substrate, slow-decaying fallen conifer wood (Nuss 1999, Holec and Kříž 2013). *Pseudoplectania nigrella* produces plectasin, a peptide antibiotic (defensin) exhibiting strong activity against *Streptococcus pneumoniae* and a very low mammalian toxicity (Mygind et al. 2005).

While considered a saprotroph, there are some reports or accessioned sequences of *Pseudoplectania* and other Sarcosomataceae species from endophyte studies (Ganley and Newcombe 2006, Stefani and Bérubé 2006, Ganley et al. 2008, U'Ren et al. 2012, Qadri et al. 2014). The possible role of endophytism in the life history of *Pseudoplectania* and other Sarcosomataceae species is unexplored; however their vernal ascospore release may be concurrent with conifer bud break, a time when succulent needles are more susceptible to infection (Harvey 1976). *Pseudoplectania* ascospores have gelatinous sheaths (Glejdura et al. 2015), a character also found in ascospores or conidia of other *Picea* endophytes isolated in this study including *Anthostomella*, *Coccomyces*, *Colpoma*, *Darkera*, *Gelasinospora*, *Lophodermium*, *Phacidium*, *Pileospora*, *Strasseria*, and *Tryblidiopsis*. Gelatinous appendages or gelatinous sheaths surrounding a spore may have several functions, including: (1) increasing the distance disseminated from the ascus by enabling ascospores to stick together; (2) absorbing free or ambient water to facilitate germination; (3) preventing loss of hydrostatic pressure within the ascus by sealing the passage of spores through the ascus opening; (4) fungus-host recognition; (5) acting as a medium through which enzymes and other molecules can migrate across; and (6) facilitating adherence to a substrate surface (Cannon and Minter 1986, Nicole et al. 1993, Viret et al. 1994, Jones 2006, Simard et al. 2013). Coprophilous fungi often possess thick gelatinous sheaths, which are thought to facilitate the adherence of spores to plant materials such as leaf lamina, and species are also sometimes found as plant endophytes (Rogers 1979, Petrini 1986). Fungi disseminating huge quantities of spores bearing adhesive appendages or

sheaths in the forest will invariably attach to plant leaves; are these fungi endophytes, “accidental endophytes”, or survivors of surface sterilization owing to thick-walled spores or improper protocol (Petrini and Fisher 1988)? For example, a single strain of the coprophilous fungus *Gelasinospora tetrasperma* was isolated as a *Picea rubens* endophyte. The presence of a spore attribute, such as an adhesive sheath or appendage, which greatly increases the incidence of prolonged contact with living plant tissues could also promote the transition from an obligate saprotrophic life history to one including an endophyte phase over evolutionary time.

Basidiomycete species were infrequently isolated as *Picea* endophytes and included taxa commonly found in mixed wood forests on decaying conifer or angiosperm wood, for example *Irpex lacteus*, *Peniophora* spp., *Schizophyllum commune*, and *Trametes versicolor* (Fig. 5.1). Ascomycete species invariably dominate foliar endophyte communities in conifer trees, with basidiomycete endophytes typically making up a smaller proportion of the observed diversity (Stefani and Bérubé 2006, Koukol et al. 2012, Larkin et al. 2012, Rajala et al. 2014, Bullington and Larkin 2015). Basidiomycete endophytes are typically neglected in studies, although Martin et al. (2015) isolated 310 basidiomycete endophyte strains from *Hevea* leaves, which clustered into 118 ITS OTUs and represented approximately 12% of the total number of isolated endophytes. Basidiomycete wood endophytes are also isolated in lower abundance compared to ascomycetes; for example, Giordano et al. (2009) and Robles et al. (2015) reported that basidiomycetes represented 12% and 26% of the total number of endophytes isolated from *Pinus sylvestris* and *Platanus acerifolia*, respectively,

despite including media selecting for basidiomycetes. Bullington and Larkin (2015) reported a negative correlation between basidiomycete and ascomycete endophytes in *Pinus monticola* seedlings. Basidiomycete endophytes detected in this study, such as *Peniophora* spp., *Schizophyllum commune*, and *Trametes versicolor*, are also reported as sapwood and root endophytes of conifers (Giordano et al. 2009, Min et al. 2014). Wood-decomposing basidiomycetes conceivably benefit from an endophytic phase by being among the first colonizers of the substrate following host senescence (Chapela and Boddy 1988).

It is conceivable that some wood-decomposing basidiomycetes may use leaves as an initial infection point, gaining entry into sapwood by way of petiole and leaf traces. Some studies of conifer endophytes suggest a degree of specificity in fungi isolated from needle petioles versus blades (Carroll and Carroll 1978, Dobranic et al. 1995). Carroll and Petrini (1983) provided evidence that conifer petiole and blade endophytes could be categorized into two distinct guilds with differing substrate utilization patterns. The authors hypothesized that petiole endophytes, which generally showed the capability to utilize a broad range of substrates including cellulose, pectin, and hemicelluloses, were vigorous decomposers or latent pathogens capable of penetrating living cells after needle senescence. Needle endophytes exhibited restricted substrate utilization capabilities, especially the inability to utilize cellulose, suggesting they were incapable of penetrating living cells and may require more simple carbon sources for active growth, possibly indicative of mutualism with the host. Carroll and Petrini (1983) also observed that many petiole endophytes rarely occurred in more distal portions of the needle.

Investigating whether leaf endophytes are generally spatially restricted to the leaf or capable of subsequently invading other host tissues may provide insight into the role of the leaf as an entry point for systemic or vascular infections.

Demystifying endophyte taxonomy

To identify the majority of culturable endophytes, significant effort must be made to collect, isolate, and sequence named-but-unsequenced or unknown field specimens with identifiable features (i.e.: sporulating reproductive structures), which are associated with the host plant or from fungi that are hypothesized to be phylogenetically-related to unidentified endophytes. Sequencing herbarium specimens, especially type specimens, is encouraged whenever possible, because type specimens provide a definitive connection between the application of a binomial name and a physical specimen. Although success rates may be poor because of competing DNA from fungal contaminants or degradation of DNA resulting from age, condition, or preservation methods, the application of new methods for NGS sequencing may assist in DNA sequencing even from herbarium specimens with degraded DNA and, when multiple specimens are pooled per run, costs can be similar to those of Sanger sequencing (Prosser et al. 2016). Additionally, some endophytes are apparently obligately biotrophic and must be sequenced directly from plant tissues, such as the Rhytismataceae species *Bifusella linearis*, *Lophophacidium dooksii* (Broders et al. 2015), and *Isthmiella faullii* (unpublished data).

The low-hanging fruit in collecting endeavors include named-but-unsequenced species associated with living or senescent host foliage. For example, Rajala et al. (2014) found that approximately one-third of detected *Picea abies* endophytes were present in both fresh and decomposing needles, indicating that many endophytes are facultative saprotrophs capable of switching trophic phases, as suggested by other authors (e.g.: Promputtha et al. 2010). In this study, the collection, isolation, and sequencing of *Micraspis acicola* was sought after because it was unrepresented by sequences and hypothesized to be a morphologically-reduced endophytic species in the Tympanidaceae, a family in which several unidentified *Picea* endophytes were tentatively placed (Fig. 5.1, see chapter 4). The collection of *M. acicola* facilitated the identification of several unknown endophyte strains that previously could not be placed within a genus or even family. Darker (1963) described *Micraspis acicola* from discoloured *Picea mariana* needles in Northern Ontario, tentatively placing it in Phacidiaceae. Since then, this species was largely forgotten; its recollection provides material allowing for phylogenetic analysis, taxonomic re-appraisal, cultural study, secondary metabolite screening, and more.

Richardson et al. (2015) described antimicrobial chlorinated benzofuran and xanthene secondary metabolites from two sterile strains of an unknown *Pinus strobus* endophyte species, referring to it as an undescribed species representing a potentially new lineage in the Massarinaceae based on ITS sequences. Broders et al. (2015) isolated strains of *Hendersonia pinicola*, a well-known secondary colonizer of pine needles and reported biocontrol agent of *Lophodermella* spp., which share 100% similar

ITS sequences with the unknown Massarinaceae sp. studied by Richardson et al. (2015). In this study, attempts to sequence herbarium specimens of *Hendersonia pinicola* failed; e.g.: the ITS sequence generated from a needle fragment of DAOM 109293 belonged to *Rhizosphaera* cf. *pini*, likely co-occurring with *H. pinicola* and *Hypodermella concolor* in the specimen. This is another example where generating reference sequences for named-but-unsequenced species from fresh field collections can provide names for unknown endophytes. A cursory survey reveals only 56% of the 260 Ascomycota species reported from *Picea* in Canada are represented by accessioned ITS sequences in GenBank (Table 2). Of these sequenced species, at least 30 are ubiquitous and not specific to *Picea* (e.g.: *Alternaria*, *Cladosporium*, *Fusarium*, *Trichoderma* spp.) and some families are disproportionately represented (e.g.: ca. 30 Ophiostomales spp.).

Many unidentified Rhytismatales endophytes were isolated in this study and field collections show the placement of the majority of these species within *Coccomyces*, *Colpoma*, or *Tryblidiopsis*. *Coccomyces* and *Colpoma* comprise ca. 230 species and are poorly represented in culture collections or GenBank. Collecting and sequencing *Picea*-associated Rhytismataceae species will provide names for unidentified endophytes and fill in the phylogenetic gaps for this large family; for example, only one *Coccomyces* sp., *C. irretitus*, is reported from *Picea* in Canada (Farr and Rossman 2016). Additionally, North American *Picea*-associated fungi known by European names may actually be distinct species that warrant description, for example the distinction between *Lophodermium piceae* and *Tryblidiopsis pinastri* on European *Picea abies* and *Lophodermium* cf. *piceae* and *Tryblidiopsis magnesii* on North American *Picea* spp.

Therrya fuckelii specimens from North America can be readily distinguished from European *T. fuckelii* specimens using the ITS barcode, suggesting the presence of a cryptic species (Figs. 5.15, 5.28). The evolutionary pathways of endophytic fungi with exclusive or narrow host preferences are likely to follow those of their hosts; therefore, genetic divergence between some North American and European fungi probably corresponds with host plant speciation. Further work with North American conifer-associated Rhytismataceae spp. with narrow host preferences and endophytic life stages will probably confirm the presence of many novel cryptic species, especially for North American Rhytismataceae spp. currently known by European names. It is hypothesized that fungi with endophytic life-histories involving broad host or substrate ranges and greater ecological plasticity, such as Mollisiaceae or Xylariaceae spp., will have a more cosmopolitan distribution.

Endophyte diversity must also include species that are viable but nonculturable, recalcitrant with regard to growth on standard media, or consist of cryptic life cycles that may not be readily elucidated using a culture-based or field approach. Such species are overlooked in most studies, including this one, but must be considered. Species diversity captured in culture-based studies can be increased by including additional media, culture conditions, or alternative isolation protocols (Prior et al. 2014), a consideration for endophyte researchers relying on standard media. Inducing sporulation of sterile endophyte cultures is laborious and enjoys variable or inconsistent success rates (Guo et al. 1998, Su et al. 2012, Knapp et al. 2015); however it may provide taxonomically informative observations when sequence data is uninformative and

provide phenotypic data that can be used to support the description of novel taxa. In cases where species comprising a genus are all represented by reference sequences or phylogenetic distinctiveness is unambiguous, authors should consider describing sterile strains (Gomes et al. 2013, Lombard et al. 2015). The description of novel species using sterile strains at least provides accessible material satisfying the reproducibility principle, and can be used to sequence additional genes or provide other insight (e.g.: chemotaxonomic characters).

The description of novel taxa known only from ENAS is controversial and currently prohibited by the International Code of Nomenclature for Algae, Fungi and Plants, but could provide a way to meaningfully incorporate the vast number of ENAS into taxonomic treatments and communicate species concepts (Hibbett and Glotzer 2011, Hibbett and Taylor 2013, Herr et al. 2015, de Beer et al. 2016). One conceivable outcome of such a system is the automated description of novel species based on ITS ENAS with little regard to the organisms in question. Relegating fungi to text strings and fungal taxonomy to an autonomous pipeline will provide names rapidly for all fungi including endophytes; however, the utility of such names is questionable. In the context of this study, describing all strains unidentifiable with GenBank reference sequences as novel taxa would certainly result in the redundant naming of named-but-unsequenced genera and species (e.g.: *Coccomyces irretitus*, *Micraspsis acicola*, *Myxocyclus cenangioides*).

Conversely, redundant names might be a small price to pay. Named species provide a means to communicate species concepts among researchers, and any relevant data associated with these new names, such as biogeography, ecology, secondary metabolite, and morphology, would remain following eventual synonymy. To some, this rationale is compelling for the haphazard naming of taxa based on their absence from GenBank and monophyly inferred from often inadequate datasets. However the rapid inflation of novel genera and species would result in taxonomic chaos, where new names can be created far more rapidly than individual names can be fixed. This amorphous taxonomic system would result in the rapid turnover of names as new relationships are continuously being re-inferred by the piecemeal addition of new sequences. The case for describing novel species based on ITS ENAS could be made for genera comprising species that are all represented by reference sequences and can be unambiguously distinguished with ITS sequences, for example *Phaeomoniella* and related genera, or the ENAS clade constituting a putatively novel *Paratritirachium* species in Nguyen et al. (2014).

The issue with delineating OTUs based on similarity thresholds highlights a substantial problem in distinguishing sister species based entirely on ITS sequences: can all species hypotheses be simplified into ITS sequences and consequently be delineated based on a consistent threshold across all lineages? The answer is unequivocally no, based on unequal intragenomic and intraspecific variability across the fungal kingdom (Nilsson et al. 2008). Fungal diversity estimates from ITS ENAS are often inflated because of sources of error such as chimeras, “deep” paralogues in multicopy markers,

and pseudogenes (Kunin et al. 2010, Lindner and Banik 2011, Větrovský et al. 2016). For example, in a mock community comprising 130 species, 275 OTUs were detected by pyrosequencing using standard ITS primers (Větrovský et al. 2016). Oliver et al. (2015) reported that depending on the clustering method and threshold of low frequency OTU removal, ITS OTU richness generated from MiSeq libraries could be exaggerated by up to 15% as a consequence of the use of non-proofreading polymerases.

We currently do not have the knowledge or means to confidently assess total species-level fungal biodiversity based solely on NGS technology. The premature application of these methods to alpha taxonomy will result in the description of “novel taxa” that are merely sequencing or bioinformatics artefacts, leaving a legacy of erroneous names for transient sequences that will confound future mycologists. The prospects of sequence-only based taxonomy are exciting and will probably prove to be the solution to rapidly categorize and name fungal biodiversity in a meaningful manner; however, a greater understanding is required prior to its adoption in the International Code of Nomenclature for Algae, Fungi and Plants. In the meantime, waiting before prematurely adopting radical new taxonomic measures based on inadequate knowledge is a more sober, if less dramatically exciting, course of action. The Code can only be modified every seven years and, with the next opportunity in 2017, some are championing for the adoption of sequence-based species descriptions in the near future (Hibbett 2016, Kõljalg et al. 2016).

The User-friendly Nordic ITS Ectomycorrhiza Database (UNITE) database allows for user-directed ITS sequence clustering into species hypotheses and the automatic or manual designation of representative sequences for subsequent species hypotheses, which are provided with unique digital object identifiers (DOIs) (Kõljalg et al. 2005, Abarenkov et al. 2010). This approach balances the need for consistent identifiers to communicate taxonomic concepts with associated reference sequences and taxonomic uncertainty. Species hypotheses and associated aggregate data serve to facilitate formal species descriptions when voucher material or cultures become available (Kõljalg et al. 2016). Good taxonomic work cannot yet be circumvented by sequences alone; new collections must be promoted and classical taxonomic knowledge and skills must be passed down to future mycologists.

It is well-established that, collectively, endophytes are ubiquitous, phylogenetically diverse, ecologically enigmatic, and in taxonomic disrepair. Progress in endophyte research requires more than a stamp-collecting approach inventorying endophytic fungi from various plant hosts. Endophytes cannot be identified in culture when taxonomically informative morphological characters are lacking or by sequences when relevant reference sequences are absent. Therefore, a different approach must be taken, one that considers the broader life-history and ecology of endophytes. The identification of culturable endophytes is straightforward but laborious; however, barcoding all unsequenced host-related or phylogenetically-relevant fungi to assist in endophyte identification is a Herculean task. Endophyte research programs must incorporate support and collaboration with taxonomists to barcode fungal taxa of

interest that are currently overlooked or hampered by the magnitude of diversity (e.g.: Helotiales and Rhytismatales). From the perspective of elucidating unknown *Picea* endophyte species, taxonomic efforts and surveys focussing on Dothidiaceae, Mollisiaceae, and Rhytismataceae genera including *Coccomyces* will be the most efficient approach.

The issues surrounding endophyte taxonomy highlight the importance of holistic species concepts consolidating disparate observations of fungi from ENAS, cultures, and field specimens (Bills et al. 2012) and the need to connect DNA sequences with meaningful names (Chaverri and Gazis 2011). Accurate identification of endophytes that are unidentifiable based on current reference sequence data is possible by connecting unknown endophyte sequences with identifiable field or herbarium specimens or by the induction of taxonomically informative morphological characters (Knapp et al. 2015, Tanney et al. 2016a). Generating endophyte reference sequences, beginning with the more commonly detected species, will make reference-based OTU clustering approaches more feasible and increase the capability of NGS studies to make more meaningful biological and ecological inferences by providing finer taxonomic resolution, and facilitate reanalysis of NGS data as new reference sequence data become available. Endophyte identification and ecological studies must go beyond ITS barcodes and consider the biology and ecology of the whole fungus.

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AUTHOR CONTRIBUTIONS

JBT conceived and designed the work. JBT acquired the data: provided strains, collections, and sequences from Canadian material; JBT provided the species descriptions, generated sequences, conducted cultural studies and phylogenetic analyses, created all photographic plates, deposited all cultures in DAOMC and specimens in DAOM. JBT analyzed and interpreted the data and taxonomic

considerations. JBT drafted the article; JBT and KAS critically revised the manuscript for content.

Conclusions and future directions

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Biodiversity and function of Acadian Forest endophytes

Foliar endophytes of *Picea rubens* and other conifers of the Acadian Forest Region are ubiquitous, phylogenetically diverse, and, prior to this thesis, taxonomically poorly characterized. Recent efforts exploring the chemodiversity of conifer endophyte strains revealed novel or known bioactive secondary metabolites exhibiting antibacterial, antifungal, and antiinsectan activities (McMullin et al. 2015, Richardson et al. 2015, Tanney et al. 2016b). Evidence of endophyte strains antagonistic to forest tree pests and pathogens provides incentive for further investigation of endemic endophytes as potential plant enhancers or producers of bioactive secondary metabolites (Clark et al. 1989, Miller et al. 2002, Sumarah et al. 2015). Greenhouse and field inoculation trials are currently underway to test the efficacy of selected endophyte strains at reducing budworm damage. The subsequent development of endophyte strains which improve tree resistance or tolerance to significant forest pathogens and pests, such as white pine blister rust and the eastern spruce budworm, will provide more effective and sustainable forest management tools.

However, the taxonomy and biology of conifer endophytes is rife with knowledge gaps. Taxonomy is a fundamental science that also provides users the means to effectively communicate and perceive patterns; the taxonomic predicament involving endophytes translates into the inability to identify and classify most endophyte strains or sequences in a meaningful way and obscures the comparison of related research.

In this thesis, an approach combining phylogenetic methods, field mycology, and culture isolation was used to identify endophytes that were unidentifiable with current ITS barcode reference sequences and cultures lacking diagnostic morphological characters. This approach was conceived because of various reports of saprotrophism in woody plant endophytes (e.g.: Sokolski et al. 2006a, Okane et al. 2008, Chaverri and Gazis 2011, Yuan and Verkley 2015, Tanney et al. 2016a, Thomas et al. 2016, U'Ren et al. 2016) under and with consideration of the foraging ascomycete hypothesis posited by Carroll (1999). The overall goals of this thesis to resolve identifications of unknown *Picea* endophyte strains with a focus on specific taxonomic groups and to demonstrate that endophyte identification can be best pursued by bridging the gap between unnamed DNA sequences and named-but-unsequenced species or novel species using a full suite of taxonomic and phylogenetic techniques, starting with field collections.

The results show the prevalence of life histories combining both endophytic and saprotrophic stages among fungi previously considered to be conifer foliar endophytes. This frequently includes reproductive stages on the foliar substrate, other host substrates, or even different hosts. Reproductive stages in turn provide the opportunity to connect endophytes with specimens containing viable spores for cultivation and taxonomically informative characters facilitating identification and classification, and allows them to be compared with preserved herbarium specimens, including types of previously described fungi. The recognition or description of unidentifiable endophytes as well as the identification of endophytic stages in fungi previously assumed to be primary or obligate saprotrophs was permitted because of such connections.

Major endophyte genera or families encountered in this study were invariably poorly characterized, often containing many species and neglected by modern treatment and sequencing efforts, for example the families Hyaloscyphaceae, Mollisiaceae, Phacidiaceae, Rhytismataceae, and Xylariaceae. Chapters one and two represent the most comprehensive sequence-based phylogenetic and taxonomic work on Mollisiaceae so far, and provide unequivocal evidence of widespread endophytism throughout this neglected family of presumed saprotrophs. This work also better delineates the taxonomic boundaries between *Phialocephala* s.s. and *Mollisia* s.l., presents an unexpected picture of the phylogenetic, morphological, and secondary metabolite diversity throughout the lineage, and provides both a roadmap and motivation for future work towards a more effective taxonomic framework. Our concept of Mollisiaceae has changed dramatically; its role in the forest ecosystem is far more complex than previously considered and species within this lineage produce significant bioactive secondary metabolites, a consideration for future screening efforts.

Diaporthe maritima was one of the most commonly isolated *Picea rubens* endophytes, and its description and the identification of its antifungal and antiinsectan secondary metabolites provides a satisfying example of multi-disciplinary collaborative endophyte research. Bioactive strains examined in chapter three are currently undergoing inoculation tests with seedlings by J.D. Irving Ltd. to investigate secondary metabolite production in planta and assess potential protectant qualities bestowed upon the host. The genotypic and phenotypic characterization of an endophyte such as

D. maritima will help elucidate its interactions with the plant host and ultimately its functional role in the forest ecosystem.

Until recently, Phacidiaceae had been more or less neglected by taxonomists for 30 years (Crous et al. 2014b, Crous et al. 2015a, Crous et al. 2015b). In this thesis, more than 100 Phacidiaceae strains representing seven species were isolated as endophytes from *Picea rubens* needles. Only strains of *Strasseria geniculata* were identifiable using the ITS barcode. *Darkera* cf. *parca* was initially identified by asexual state morphological characters formed in vitro and later by comparison with the ITS barcode generated from the holotype. Morphological and phylogenetic analyses resulted in the description of two novel *Phacidium* species, *P. dicosmum* and *P. faxum*, and three monotype genera to accommodate distinct species: *Calvophomopsis rubensicola*, *Cornibusella unguolata*, and *Gloeopycnis protuberans*. Previously, few Phacidiaceae species were reported from Canadian *Picea* spp., e.g.: *Ceuthospora pithyophila*, *Darkera parca*, *Pseudophacidium garmanii*, *P. piceae*, and *Strasseria geniculata* (Farr and Rossman 2016). This work greatly increases the known biodiversity of Phacidiaceae endophytes associated with *Picea* and provides a means to identify them using both cultural characters and DNA reference sequences. Many Phacidiaceae species are considered pathogens; the identification of endophytic species warrants further investigation into host interactions and might provide interesting examples of a continuum between endophyte and pathogen from the same colonization event.

The overall biodiversity of culturable *Picea* endophytes is high and comprises many unidentified or novel species. The last chapter in this thesis included a broad

survey of *Picea* endophyte biodiversity across diverse lineages and provided examples of connections between endophytes and their saprotrophic reproductive stages, which facilitated more precise identification. These connections also offered evidence of more complex endophyte life histories, many of which are not constrained to the host needle. Culturable endophytes of *Picea* needles exhibited varying degrees of host and host-substrate specificity or preference. Some species were exclusive to the host needles, probably causing asymptomatic infections until the occurrence of natural or precipitated death initiates extensive colonization and subsequent reproduction (i.e.: pioneer decomposers). For example, *Lophodermium* cf. *piceae* exhibits an endophyte-saprotroph life history and is spatially restricted to *Picea* needles.

Alternatively, fungi can endophytically colonize additional host tissues and produce reproductive structures on specific non-foliar tissues. Examples are *Tryblidiopsis magnesii*, an endophyte that produces ascomata on self-pruned branches, and *Coccomyces irretitus*, an endophyte that produces ascomata on bark. *Phialocephala scopiformis* was originally described as a branch endophyte of *Picea abies* (Kowalski and Kehr 1995). In this thesis, *P. scopiformis* was commonly isolated as a foliar endophyte and apothecia were only found on decaying *Picea* branches.

Other fungi are able to infect a broad range of hosts as endophytes but reproduce on alternate hosts with some narrow preference observed, such as *Xylaria ellisii* and *Nemania* spp. Several studies have also observed the presence of sporulating endophytes outside of their plant host (e.g.: Okane et al. 2008, Chaverri and Gazis 2011,

Yuan and Verkley 2015, Tanney et al. 2016a, Thomas et al. 2016, U'Ren et al. 2016), indicating many endophytes are capable of functioning as either endophytes or saprotrophs.

Revisiting the foraging ascomycete hypothesis

Carroll (1999) introduced the foraging ascomycete hypothesis to explain the ubiquity of endophyte infections caused by saprotrophic Xylariaceae species (e.g.: Petrini and Petrini 1985, Petrini et al. 1995, Osono et al. 2013). This hypothesis proposes that for some saprotrophic fungi, endophytism is a secondary life history strategy that facilitates persistence and dispersal in the absence of substrates suitable for reproduction (i.e. a primary host). For example, Thomas et al. (2016) isolated Xylariaceae endophytes from 38 tree species in a tropical cloud forest and connected all five *Xylaria* endophytes with saprotrophic (stromatal) life stages. *Hypoxylon pulicicidum*, a pantropical endophyte producing antiinsectan nodulisporic acids, was detected in several independent studies and eventually described following the collection of stromata on decaying wood (Bills et al. 2012).

The results from this thesis provide corroborative evidence that many conifer needle endophytes have corresponding saprotroph stages on primary substrates. The number of isolated conifer endophytes detected or known to occur as saprotrophs occurring reproductively on alternative hosts or host substrates is striking: *Annulohypoxylon multiforme*, *Cenangium* sp., *Coccomyces irretitus*, *C. strobi*, *Colpoma crispum*, *Colpoma* sp., *Dermea peckiana*, *Diaporthe maritima*, *Dwayaangam colodena*,

Hyphodiscus sp., *Irpex lacteus*, *Lachnum* spp. *Lophiostoma corticola*, *Melanconis alni*, *Mollisia novobrunswickia*, *M. melaleuca*, *M. nigrescens*, *Peniophora cinerea*, *Pezicula cinnamomea*, *P. sporulosa*, *Peziza varia*, *Phialocephala amethystea*, *P. helena*, *P. nodosa*, *P. piceae*, *P. scopiformis*, *Pileospora piceae*, *Schizophyllum commune*, *Trametes versicolor*, *Tryblidiopsis magnesii*, *Xylaria ellisii*, and others. Endophytism is clearly not an obligate life strategy and applying this knowledge to our taxonomic approach will help resolve issues identifying and classifying endophytes.

These observations provide support for the foraging ascomycete hypothesis, which in turn provides a compelling rationale for the persistence and prevalence of horizontally-transmitted endophytism throughout diverse lineages of Dikarya. A fungus capable of resisting or avoiding host defenses by means of an endophytic infection is afforded a refuge buffering it from a barrage of stresses that it would otherwise face in its saprotrophic stage, including starvation, UV radiation, drought, microbial competition, and mycophagy, and the fungus is also conferred a source of nutrients and a vehicle for persistence and dispersal (Thomas et al. 2016). *Picea* trees retain needles for several years to over a decade depending on environmental conditions (Hom and Oechel 1983, Reich et al. 1996, Kayama et al. 2002), presenting a refuge amenable for long-term persistence by endophytes.

While foliage may provide a suitable substrate for reproduction and dispersal following senescence or death, for example the production of ascomata and conidiomata by *Lophodermium* cf. *piceae* and conidiomata and sclerotia by *Penicillium*

sect. *Thysanophora* spp., Thomas et al. (2016) demonstrated the direct transmission of endophytes from leaves to woody substrates by means of physical contact. Thus the shedding of foliage may act as a dispersal mechanism for saprotrophic endophytes capable of growing on substrates present in the forest floor. This mode of transmission from leaf-to-wood could explain the occurrence of wood-decaying basidiomycetes as foliar endophytes (e.g.: *Trametes versicolor*). The role of insects as vectors should also be considered; for example, many *Picea* endophytes produce conidial droplets that can conceivably be spread to other needles by insects (e.g.: *Calvophomopsis rubensicola*, *Darkera parca*, *Diaporthe maritima*, Dothideales spp., *Gloeopycnis protuberans*, *Pezicula* spp., *Phacidium* spp., *Pileospora piceae*, *Strasseria geniculata*). Rhytismataceae species typically form conidiomata from which droplets of conidia are produced, often considered spermatia because of their occurrence before ascomata and their inability to germinate on standard media (e.g.: as observed in conidia of *Lophodermium* cf. *piceae*, *Tryblidiopsis magnesii*) (Osorio and Stephan 1991). The dispersal of spermatia is assumed to be by rain splash and insects.

Collectively, endophytes interact with their hosts along the endosymbiont-pathogen continuum, depending on a balance of antagonisms involving the immediate phenotype status of the fungus and host (Schulz and Boyle 2005). It is this phenotypic plasticity, combined with phylogenetic diversity, that makes horizontally-transmitted endophytes so difficult to define and categorize conceptually, taxonomically, and ecologically (e.g.: Rodriguez et al. 2009), especially given the predilection of research to focus on the endophytic status of a fungus and not consider its overall life history (e.g.:

Arnold 2007, Higgins et al. 2007). Mounting evidence reveals both endophytic and saprotrophic life stages in phylogenetically diverse endophytes; the complete life history of these endophytes must be considered when investigating endophyte communities (Peršoh et al. 2010).

In this sense, a reconsideration of Wilson's (1995) definition of an endophyte presented in the thesis introduction is warranted: "Endophytes are fungi which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease". The adoption of the more inclusive definition of endophytism in this thesis, as provided by Wilson (1995), proved constructive. The prevalence of combined saprotrophic-endophytic life histories of *Picea* endophytes supports the notion that endophytism is best considered a life history strategy that occupies all or part the fungus' life, rather than endophytism being an obligate mode of trophism.

Overall, this thesis provides an example of a holistic approach for addressing fundamental taxonomic issues in a phylogenetically diverse and heterogeneous group of fungi occupying a significant but poorly understood ecological functional group. It is also a significant contribution to the understanding of endophyte biodiversity in the Acadian Forest Region: four novel genera and 21 novel species were described, with representative strains undergoing screening and bioassays to discover bioactive secondary metabolites of potential interest. Yet this represents a small number of the potentially novel species encountered in the Acadian Forest Region. Collecting efforts

and taxonomic studies focussing on species in families such as Dothidiaceae, Mollisiaceae, Rhytismataceae, and Xylariaceae will be the most efficient strategy to elucidate a good portion of the endophyte biodiversity of *Picea*. This approach will simultaneously identify unknown endophytes and ameliorate phylogenetic gaps in poorly sampled taxonomic groups.

The past and the future

Forests are critical to the ecological and economic integrity of Canada, yet relatively little is known about the overall biodiversity of the fungal Kingdom within these communities, especially microfungi and ascomycetes. Excellent mycological taxonomic research was conducted in Canadian forests in the past but this waned about 30 years ago (e.g.: Groves 1952, Darker 1967). Consequently, reports or collections of many forest-associated fungi described in the 20th century remain scant. The result is poorly-understood species that remain unsequenced and unrepresented by viable cultures, effectively hiding them from many modern researchers. Additionally, much of the previous research was understandably focused on pathogens, with little consideration to the essentially unknown tree endophytes. Consequently, there is a large body of taxonomic literature available that is assumed to be related to forest pathology but is also likely relevant for endophyte research.

Sequencing technology is progressing rapidly, costs per base pair are decreasing annually, and bioinformatics pipelines are becoming more effective at dealing with the deluge of sequences from ecological studies. These tools hold enormous potential for

increasing our understanding of the fungal component of our planet, yet taxonomists must do more than just feed the machines. Considering the whole fungus interacting within its natural habitat will provide more biological insight and a better taxonomic framework versus a mechanical taxonomic approach that reduces fungi to DNA barcodes.

The development and adoption of new concepts and tools sometimes results in a tendency to allow old knowledge to be swept away, because it is perceived as archaic and irrelevant. However, we risk repeating the mistakes of scientific history and losing important knowledge and concepts by neglecting work conducted prior to the most recent paradigm shift. The application of new technology to old knowledge can interpret and test previous concepts at a finer resolution or accuracy and circumvent the continual reinvention of the wheel at the cost of finite resources. Revisiting the work, specimens, and cultures of previous mycologists with new tools and insight will advance our understanding of forest mycology and maintain a connection and commensurability with past knowledge.

The results from this thesis should therefore provide convincing support for significantly expanding field collecting efforts and the mining of invaluable resources such as herbaria and culture collections. Classical taxonomic knowledge is needed now more than ever and the synergistic marriage of both classical taxonomy and modern molecular methods leads to significance advances in mycology.

Furthermore, culturing efforts provide material that can be used to explore genomics, metabolomics, and other aspects of fungi. The identification of novel or known bioactive secondary metabolites from conifer endophytes of the Acadian Forest Region is possible because of such culturing endeavors. Understanding the life history and taxonomy of fungi that produce bioactive metabolites of interest also facilitates the development of potential biological control agents or plant enhancers. For example, finding higher rugulosin producers requires access to a large number of *Phialocephala scopiformis* strains. Previously, isolating *P. scopiformis* endophytes was a more-or-less chance occurrence; however, we can now generate countless single ascospore *P. scopiformis* strains by collecting apothecia occurring on decaying fallen *Picea rubens* branches. Further culture- or inoculation-based work should include connecting genomic data with phenotypic data to identify genetic signatures of endophytism and the mechanism enabling endophyte-saprotroph switching.

This multi-disciplinary investigation of endophytes has already resulted in the discovery of novel species and bioactive secondary metabolites and assists the ongoing development of plant enhancing agents to combat forest pests and pathogens. Canadian forests have been devastated by the introduction of invasive alien pests and pathogens in the last century alone, with an increase in invasive species establishment expected from globalization and climate change (Early et al. 2016). The decimation of dominant forest trees, such as *Castanea dentata*, *Ulmus americana*, and *Fraxinus* species, severely disrupts the landscape and consequently the ecological and economic integrity of Canada. However, the true impact of these events on the landscape and

biodiversity cannot be properly assessed or fathomed without elucidating the affected host plant associates, including endophytes and other components of the plant microbiome. Endemic endophytes are an overlooked source of forest biodiversity in Canada and may provide tools that protect managed forests from destructive native and alien species.

Furthermore, just as old growth *Picea rubens* forests are reservoirs of tree genetic diversity and possible superior seed sources (Mosseler et al. 2003b), these same old growth forests contain a great biodiversity of horizontally-transmitted endophytes and represent important sources of potentially commercially-viable endophyte strains. This provides a pragmatic incentive to protect old growth forests and ascribes additional value to the retention of trees and coarse woody debris, which may serve as sources of endophyte inoculum for regenerating forests or for future discoveries.

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