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Diversity, Systematics, and Ecology of Bryophilous Fungi

by

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Abstract

In this thesis, a literature review identified a lack of culture-based studies of the taxonomic diversity and ecological function among bryophilous fungi. Therefore, this research examined the diversity of fungi associated with some temperate bryophytes from Canada using a culture-based approach that identified isolates by morphological and rDNA ITS sequence characters. Seventy-one operational taxonomic units or species were identified, of which 34 had not been previously reported from mosses. *Cladophialophora minutissima* sp. nov., and *Atradidymella muscivora* gen. et sp. nov. and its anamorph, *Phoma muscivora* sp. nov. were described. *Cladophialophora minutissima, A. muscivora*, and *Coniochaeta velutina* were selected for characterization of their ecological functions by enzyme profiling and observation of the host-fungus interface by light and scanning electron microscopy and using *Funaria hygrometrica* as a model host. *Cladophialophora minutissima* is a latent saprobe of mosses, and infected hosts remain asymptomatic. *Atradidymella muscivora* is a generalist pathogen that exhibits specific adaptations to bryophyte hosts, including the formation of pycnidia and minute stroma within single host cells. A new mode of nutrition among bryophilous fungi was described in *Atradidymella muscivora*, whereby the fungus intercepts nutrients that would normally be destined for the host's developing gemmae. The opportunistic vascular plant pathogen *Coniochaeta velutina* was weakly parasitic or saprobic; it did not penetrate living cells, and instead persisted on senescent or moribund rhizoids, where it formed both superficial perithecia and a *Lecythophora* anamorph. This anamorph displayed a previously undescribed pattern of sporulation in which conidia formed within the rhizoids, and dispersal occurred only upon host rupture. The ability of a vascular

plant pathogen to persist on a moss host suggests bryophytes may represent an additional reservoir of disease inoculum in temperate ecosystems.

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Chapter 1

Diversity and ecology of bryophilous fungi

Introduction

Fungi from all phyla of the kingdom Mycota have been reported to colonize plant tissues, and have been ascribed ecological roles as endophytes, parasites, pathogens, mutualists, and commensals within living tissues, as well as acting as saprobes, decomposing moribund and senescent plant material. The vast majority of research on fungi associated with plants has focused on vascular plant lineages and relatively little, by comparison, is known about fungi associated with non-vascular plants.

In the context of this thesis, 'bryophytes' is used to refer to mosses (Bryophyta), a lineage of non-vascular plants present on all continents in habitats ranging from deserts to forests to aquatic settings. Bryophytes colonize a diverse array of substrata including rock, soil, trees (as epiphytes), and decomposing plant and animal matter. It is well documented that bryophytes are an integral component of their ecosystems, playing roles in photosynthetic production, microhabitat creation, nutrient cycling, and pedogenesis, and they are also dominant components of the vegetation of several biomes, including the boreal forest, peat forming wetlands, and the tundra of the polar regions (Smith 1982, Schofield 1985). Despite the global abundance and ecological importance of bryophytes very little is known about their interactions with the microbial community of their ecosystems, or the nature of the fungal communities inhabiting both live bryophyte hosts and senescent or dead moss substrata. While bryophytes share basic similarities with vascular plants such as cellulosic cell walls, and specific metabolic functions

(photosynthesis), they display significant differences in anatomy, ecology, and life history (Smith 1982, Schofield 1985), and as such, it is unlikely the diversity and ecological function of bryophilous fungi can be extrapolated from our current knowledge of fungi associated with vascular plants. In bryophytes, the gametophyte generation is dominant, and the sporophyte is dependent on it for its nutrition. While the gametophyte is differentiated into stems and leaves, much like vascular plants, the leaves of bryophytes are anatomically extremely simple, and are only one cell thick across the lamina, with the exception of a few taxonomic groups. Furthermore, bryophytes lack absorptive root structures and vascular tissues, instead producing filamentous rhizoids that function in anchoring the gametophyte to its substrate, and relying primarily on capillary conduction of water and absorption of moisture and nutrients over the entire surface of the thallus (Schofield 1985). Finally, most bryophytes are poikilohydric, and have life history strategies that are oriented to dessication tolerance, as opposed to the dessication avoidance tactics that are observed in many vascular plants (Smith 1982). Given the specialized anatomy and ecology of mosses, it would be expected that they host a unique suite of fungi suited to the microenvironment of the bryophyte host.

Although reports of fungi associating with bryophytes date back to the late 18th century (Hedwig 1789), research on bryophilous fungi has been mostly scattered, inconsistent, and without focus. Currently, the diversity of fungi associated with bryophytes is poorly circumscribed and species are generally known only from morphological descriptions of herbarium material, and the ecological function of fungi relative to bryophyte hosts and substrata has scarcely been investigated.

Taxonomic Diversity Among Bryophilous Fungi

Comprehensive studies of the diversity of fungi associated with bryophyte hosts and substrates have not been undertaken, nor does there exist a sufficiently large body of work on the topic to indirectly circumscribe the group. Kost (1988) and Felix (1988) provide summaries of known bryophilous basidiomycetes based on fruiting bodies associated with mosses, noting species from some 16 families in 4 orders. The biodiversity of microascomycetes that form sexual fruiting structures on moss hosts has been studied in detail, and several hundred species of ascomycete associates of bryophytes have been identified based on morphological characters displayed on collections of host mosses (Racovitza 1959, Felix 1988, Döbbeler 1997). However, frequent descriptions of new species (Döbbeler and Treibel 2000, Döbbeler 2006, Döbbeler 2007) discovered by studying bryophilous fungi in this fashion suggest the biodiversity of fruiting ascomycetes on bryophyte hosts still has not been thoroughly described. Only a very few studies in specific moss dominated habitats [boreal bogs (Thormann et al. 2001) and Antarctic tundra(Kerry 1990, McRae and Seppelt 1999, Tosi 2002)] have used culturing methods to investigate the taxonomic diversity of bryophilous fungi, although the limited species lists generated by these investigations have identified additional diversity among bryophilous fungi, and several new species (Tsuneda 2000, Rice and Currah 2006) have been described among fungi cultured from mosses and moss substrates.

The culturing of fungi from bryophyte hosts and substrates has also elucidated the existence of associates of mosses that are sterile in culture (Thormann 2001), suggesting

additional cryptic taxonomic diversity may be present. Despite the popularity of combining culturing and molecular techniques, or employing culture-free molecular techniques to investigate the taxonomic diversity of fungi, particularly those that are cryptic (Arnold et al. 2007 and references contained), molecular techniques have never been employed to investigate the taxonomic diversity of bryophilous fungi. Given the complementary nature of morphological and molecular (both with cultures and culture-free) approaches to assessing within-community diversity an approximation of the diversity of bryophilous fungi will only be achieved by a combination of the three approaches.

Ecological Function of Bryophilous Fungi

A variety of ecological functions have been postulated for different bryophilous fungi, including as pathogens, parasites, saprobes, and commensals, but few of these have been studied in any detail. Although mycorrhizal fungi have been reported to colonize bryophytes, these associations appear to be only saprobic, and functional mycorrhizal associations have not been observed (During and van Tooren 1990). Non-mycorrhizal fungi have also been identified degrading bryophyte substrates, although the mechanisms by which degradation occurs are not well understood for most of these saprobes (Racovitza 1959, Thormann et al. 2002, Rice et al. 2006). A wide variety of parasites and pathogens have been reported from moss hosts (Racovitza 1959, Kost 1988, Döbbeler 1997), primarily as fruiting bodies associated with moribund gametophytes. While symptoms of disease and infection caused by these fungi are well documented, disease

etiology and host-fungus interfaces have generally been described only cursorily. As such, it is not well understood how fungal pathogenesis in bryophytes compares to vascular plants, although observations of similar pathogenesis structures (Döbbeler 1997) and homology in disease resistance genes between vascular plants and bryophytes (Akita and Valkonen 2002, Andersson et al. 2005) suggests pathogenic processes may be similar in both hosts. Because only a handful of studies have isolated bryophilous fungi in culture (Kerry 1990, McRae and Seppelt 1999, Thormann et al. 2001, Tosi et al. 2002), cryptic fungi that do not obviously sporulate at their host's surface, including endophytes, are virtually unknown from moss hosts, and their ecological significance remains unidentified. Although bryophilous fungi have been reported to have a variety of ecological functions, they generally have not been characterized in detail, and the mechanisms driving the interactions between the fungus and its bryophyte host or substrate are not well known.

Research Rationale and Objectives

The first objective of this thesis was to address the lack of comprehensive information regarding bryophilous fungi and the nature of their interactions with bryophyte hosts. A review of both bryological and mycological literature was undertaken to provide an overview of those interactions that have been observed or postulated between bryophytes and the diverse array of fungi that colonize them. The following areas were specifically addressed: i) the taxonomic diversity already described among bryophilous fungi, ii) the anatomy of host-fungus interfaces among bryophilous fungi, iii) potential ecological functions of bryophilous fungi as pathogens, parasites, saprobes, and commensals, and iv)

identification of ecological processes that bryophilous fungi may play a role in within the ecosystems they inhabit. A comprehensive review of the literature on these particular topics was needed to delimit gaps in the current knowledge of bryophilous fungi and to provide direction for further research, and a summary of this information can be found in Chapter 2.

'Comprehensive' studies of the diversity of fungi associated with mosses have been heavily biased towards those fungi that form identifiable sexual or asexual fruiting structures in association with or on the surface of moss gametophytes and sporophytes, to the exclusion of any cryptic or non-sporulating fungi present on the bryophyte host. These studies suggest mosses whose gametophytes offer a greater number of microniches for fungal colonization are host to a greater taxonomic diversity of bryophilous fungi (Döbbeler 2002). The second research objective focused on using additional culturing and molecular techniques to approximate diversity among cryptic fungal associates of different moss hosts, particularly in light of sterile isolates. A summary of those fungi isolated from selected host moss species, both sporulating and sterile, can be found in Chapter 3, and two of these are described as new species in Chapters 4 and 5.

The third research objective addressed the paucity of functional studies that have been conducted on bryophilous fungi, and their bias towards basidiomycetous fungi. Despite many records of pathogenesis and parasitism among bryophilious fungi, few have been isolated in culture, and the disease etiology of fungi pathogenizing bryophyte hosts has only been described in detail for four fungal species [*Scleroconidioma sphagnicola* (Tsuneda et al. 2001a), *Tephrocybe palustris* (Untiedt and Muller 1985), *Leptoglossum*

retirugum (Hassel and Kost 1998), and *Eocronartium muscicola* (Boehm and McLaughlin 1988)] of which three are basidiomycetes. Similarly, cell wall degradation and the extracellular enzymes produced by bryophilous fungi have been characterized for only a few fungi isolated from mosses, and these studies have been mostly limited to fungi associated with peat (Thormann et al. 2001, Tsuneda et al. 2001b, Thormann et al. 2002, Rice et al. 2006). The third research objective was to identify and culture saprobic, pathogenic, and parasitic bryophilous fungi to allow for *in vitro* investigation of extracellular enzyme production, the host fungus interface, and infection etiology. Chapters 6 and 7 describe in detail the interactions between *Atradidymella muscivora* and *Coniochaeta velutina* with bryophyte hosts.

The three research objectives described above were undertaken with the overarching goal of providing a more indepth examination of the taxonomic diversity and range of ecological functions present among bryophilous fungi. The research described above is put into the context of current knowledge of bryophilous fungi, and directions for future research are identified in Chapter 8.

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Chapter 2
Interactions between mosses (Bryophyta) and fungi[1]

Introduction

Associations between mosses and fungi were long thought to be uncommon and rare (Grasso and Scheirer 1981), a misconception that can be attributed to the study of moss specimens in herbaria that are collected with a bias for healthy plants, an abundance of minute parasitic fungi causing few macroscopic symptoms on their hosts, and a lack of cooperation between bryologists and mycologists. In truth, bryophytes are host to diverse fungal communities that have remained poorly characterized, in part, as a result of this misconception.

Although research has been sporadic and often resulting in lists of species forming associations, zygomycetes, ascomycetes, basidiomycetes, and oomycetes have been reported as associates of a taxonomically diverse array of mosses. Racovitza (1959) and Felix (1988) have provided comprehensive reviews of the species diversity among sporulating fungi reportedly associated with bryophytes, while Kost (1988) and Döbbeler (1997) have briefly reviewed the biodiversity of bryophilous basidiomycetes and ascomycetes, respectively. Culture-based studies of sporulating microfungi associated with mosses in polar tundra (Kerry 1990, Möller and Dreyfuss 1996, McRae and Seppelt 1999, Tosi et al. 2002) and boreal wetland (Thormann et al. 2001) habitats have revealed additional taxonomic diversity among bryophilous fungi, but have not addressed those sterile cultures isolated.

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[1] A version of this chapter has been published. Davey, M.L. and R.S. Currah. 2006. Canadian Journal of Botany 84:1509-1519.

Although the taxonomic diversity of those bryophilous fungi that sporulate readily *in vitro* or *in vivo* has been well described, ecological interactions between these fungi and their moss hosts have remained largely unstudied from morphological, functional, and ecological approaches. This chapter examines aspects of the various types of associations between mosses and fungi and suggests directions for future research.

Pathogenic Interactions

Although mosses do not produce 'pathogen targets' such as the nutrient rich storage structures or specialized transport tissues rich in photosynthetic products found in vascular plants, fungal pathogenesis of mosses is being reported with increasing frequency. Unlike vascular plant pathogens, the morphology, physiology, ecology, and etiology of bryophyte pathogens and their host-parasite interactions are not well characterized. Further investigation of these relationships is needed to determine if current models of vascular plant pathogenesis are transferable to bryophyte systems.

Symptoms of fungal pathogenesis in vascular plants can involve chlorosis and necrosis of the leaves and stem, wilting, leaf drop, and significant, abnormal anatomical changes in individual plants (Agrios 1997); fungal pathogens of bryophytes can be detected by the macroscopic, black, brown, or yellow necrotic and chlorotic patches they cause in otherwise healthy stands of moss gametophytes (Fig. 2-1). Fungi such as *Tephrocybe palustris* (Peck) Donk (Redhead 1981, Kost 1988), *Bryoscyphus dicrani* (Ade & Hohn.) Spooner (Henderson 1972), *Scleroconidioma sphagnicola* Tsuneda, Currah, & Thormann (Tsuneda et al. 2000), *Acrospermum adeanum* Hohn. (Döbbeler

1979a), *Arrhenia retiruga* (Bull) Redhead (Hassel and Kost 1998) and *Lizonia baldinii* (Pir.) Dobb. (Döbbeler 2003) form small, roughly circular areas of dead and moribund moss gametophytes, while *Thyronectria antarctica* var. *hyperantarctica* Hawksw. (Hawksworth 1973) and *Pythium ultimum* var. *ultimum* Trow. (Hoshino et al. 1999), as well as unidentified ascomycetous and basidiomycetous pathogens of polar mosses cause the formation of single or concentric rings of dead moss gametophytes several metres in diameter (Wilson 1951, Longton 1973, Fenton 1983). While the general symptoms of chlorosis and necrosis, followed by gametophyte death, are common to all known bryophilous pathogens (Wilson, 1951, Redhead 1981, Untiedt and Muller 1985, Kost 1988, Tsuneda et al. 2000, Döbbeler 2003), mechanisms of host cell penetration and disruption, etiology and disease dissemination, and host responses to infection vary.

In vascular plants, host penetration is often facilitated by appressoria and (or) penetration pegs that use mechanical pressure and lytic enzymes, respectively, to breach the host cell wall (Agrios 1997). In bryopathogenic fungi, appressoria are uncommon; instead, penetration peg formation and enzymatic digestion of the host cell wall appears to facilitate intracellular penetration. The host-cell penetration mechanism of the agarics *Arrhenia retiruga* and *Tephrocybe palustris* (Tricholomataceae, Agaricales) are perhaps the best characterized. *Tephrocybe palustris* forms penetration pegs that locally produce pectinases to digest the middle lamella between *Sphagnum* leaf cells and facilitate entry into both chlorophyllous and hyaline cells (Redhead 1981, Untiedt and Muller 1985, Simon 1987, During and van Tooren 1990). Similarly, *Arrhenia retiruga* enzymatically produces channels that allow hyphae to intrude into host cells (Hassel and Kost 1998).

Other necrotrophic pathogens, such as the perithecial ascomycetes *Nectria mnii* Dobb. (Nectriaceae, Hypocreales) (Döbbeler 1988) and *Lizonia baldinii* (Pseudoperisporiaceae) (Döbbeler 2003), the ascomycetous hyphomycete *Scleroconidioma sphagnicola* (Dothideales) (Tsuneda et al. 2001a), and basidiomycetous pathogens causing concentric 'fairy rings' in stands of Antarctic moss gametophytes (Wilson 1951) also lack distinct appressoria and therefore likely produce extracellular enzymes to facilitate host cell penetration, although these are unidentified. Moss cell walls are composed of heterogeneous, polymorphic biopolymers composed of both cellulosic and polyphenolic components (Popper and Fry 2003, Lee et al. 2005) allowing pathogenic fungi to use a diverse array of extracellular enzymes to effect host cell penetration. The production of extracellular, wall digesting enzymes also appears to be under spatio-temporal regulation in a species-specific manner. Many species, such as *Tephrocybe palustris* (Untiedt and Muller 1981) and *Nectria mnii* (Döbbeler 1988), penetrate host cells using fine penetration pegs, and cell wall digestion occurs only at the advancing tip of the peg; further digestion of the host cell wall is not observed (Redhead 1981, Untiedt and Muller 1985). In contrast, *Scleroconidioma sphagnicola* hyphae grow throughout the cell wall, digesting wall components and creating characteristic wavy deformations of the cell wall and localized voids surrounding intracellular hyphae (Figs. 2-2, 2-3) (Tsuneda et al. 2001a). These differences suggest bryophilous pathogens exploit different nutritional microniches within the gametophyte; those fungi exhibiting little cell wall degradation primarily exploit easily assimilable photosynthetic derivatives while others exhibiting significant cell wall degradation derive nutrition from both structural and storage

components of the cell. Classes of degradative enzymes present in bryopathogenic species may reflect evolutionary history; the agarics *Arrhenia* and *Rimbachia* are thought to have arisen from soil and wood degrading species. The enzyme suites of these species facilitated a jump to a bryophilous host whose cell wall chemical composition is similar to that of peaty soils and wood (Redhead, 1984).

Although pathogenicity is, by definition, the 'ability of a parasite to interfere with one or more of the essential functions of the plant' (Agrios 1997), little is known about the mechanisms by which fungal pathogens of bryophytes disrupt their hosts' cells to cause necrosis. *Tephrocybe palustris* preferentially penetrates living, chlorophyllous leaf cells of *Sphagnum fallax* (Klinggr.) Klinggr. resulting in deterioration of the protoplast (Untiedt and Muller 1985). Similarly, *Scleroconidioma sphagnicola* selectively invades chlorophyllous leaf cells of *Sphagnum fuscum* resulting in chloroplast degeneration and cell death (Tsuneda et al. 2001a). In both cases, host cells collapsed and died in the absence of penetrating hyphae, leading to the hypothesis that the fungi are producing extracellular toxins; however, it remains unknown whether the necrosis is due to a hypersensitive host response, or secreted toxins and the cellular structures or processes they affect. Similarly, *Phyllosticta tetraplodontis* Lebedeva causes browning and chlorosis of the sporophytic tissues of *Tetraplodon*, eventually causing the capsules to fall off. The method of cell disruption and degradation for this organism is unknown (Racovitza 1959). Furthermore, it is unclear whether these fungi are capable of deriving nutrients from live cells, or whether their primary nutrition is from the breakdown products in dead cells. *Lizonia baldinii* forms perithecia in dense pseudostromatic

aggregations within the antheridial cups of male gametophytes of polytrichaceous mosses, with the vegetative mycelium enveloping the innermost involucral bracts and penetrating the central conducting strand of hydroids and leptoids, resulting in gradual death of the plant from the apex down (Parriat and Moreau 1954, Döbbeler 2003). Although membrane hydrolysis has been observed (Racovitza 1959), it is unknown if the necrosis is attributable to protoplast rupture, the intracellular spread of hyphae, a hypersensitive response by the moss, or the production of toxins which are carried in the stem's central conducting strand. *Nectria mnii*, also a perithecial ascomycete pathogen of polytrichaceous mosses, produces networks of pseudoparenchymatous hyphae that displace cell contents throughout stem tissues outside the central conducting strand (Döbbeler 1988). How the fungus is excluded from the central conducting strand and whether host cells are killed by fungal products, or if host protoplasts are merely displaced by the invading hyphae are unknown. Potentially, the necrosis and host death associated with fungal pathogens of bryophytes could be attributed to a variety of mechanisms including lytic enzymes, toxins, mechanical displacement of cell contents and organelles, and hypersensitive host responses.

In pathogenesis of vascular plants, effective propagule dissemination and perennation are important, as host plants are killed or often unavailable for part of the year. Typically, the asexual state of the fungus usually produces large amounts of inoculum that result in disease dissemination, while sexual structures preserve genetic diversity, and either resistant asexual or sexual spores or asexual perennating structures, such as microsclerotia, facilitate the survival of unfavorable conditions (Agrios 1997).

Although moss hosts are available year round, fungal pathogens must still survive the prolonged periods of desiccation, freezing and UV irradiation that can occur in moss-dominated ecosystems. Although moss pathogens are known to have a variety of dispersal mechanisms, their life cycles are poorly known, and consequently, the disease etiology of most moss pathogens is unknown. The hyphomycete *Scleroconidioma sphagnicola* produces minute, grain-like microsclerotia that may act as disseminative or perennating structures or may develop into conidiomata that produce conidia adapted to water or insect dispersal (Figs. 2-4, 2-5) (Tsuneda et al. 2001a, Tsuneda et al. 2001b). The perithecial ascomycetes *Nectria mnii* (Döbbeler 1988) and *Lizonia baldinii* (Döbbeler 2003) produce only ascomata and have no known asexual state and do not form asexual perennating structures. Meanwhile, *Acrospermum adeanum* (Acrospermaceae, Xylariales) produces both a perithecial sexual state and a conidial asexual state on the leaves of Bryalean and Hypnalean gametophytes, but does not form any perennating structures (Döbbeler 1979a). In bryopathogenic species with no known sexual stage it is unclear how recombination and genetic diversity are maintained to ensure the pathogen can cope with the evolution of host defences. Similarly, the etiology and dissemination of disease in pathogens of mosses with no known asexual states are poorly understood, as are the mechanisms by which pathogens lacking perennating structures are able to survive prolonged periods of desiccation, freezing, and UV irradiation on moss dominated ecosystems. Life-cycle stages potentially important to disease dissemination and etiology of these pathogens may have been missed because they were absent when the bryophytes were harvested, or isolation in culture was

unsuccessful. There is also a preoccupation with finding sexual fruiting bodies when moribund mosses are examined.

Bryophilous pathogens show structural and functional adaptation for their pathogenic habit. For example, the agaric *Tephrocybe palustris*, and the apothecial *Bryoscyphus dicrani* (Helotiaceae, Helotiales) have thin, delicate stipes that are stabilized and supported by the surrounding moss cushion, maintaining the fertile portion of the fruit body in an upright, elevated position that is favourable for spore dispersal (Henderson 1972, Kost 1988). Meanwhile, *Lizonia baldinii* and *Durella polytrichina* (Karst.) Racov. (Helotiaceae, Helotiales) produce ascomata at the apex of *Polytrichum* gametophytes within the antheridial cups, optimizing the opportunity for wind dispersal of its ascospores (Racovitza 1946, Döbbeler 2003). The agaric *Arrhenia retiruga* employs a similar strategy by forming basidiocarps directly on the sporophytes of *Brachythecium rutabulum* (Hedw.) Br. Eur. (Hassel and Kost 1998). Similarly, *Bryochiton* (Pseudoperisporiaceae, *incertae sedis*, Dothideomycetidae) forms ascocarps on the abaxial side of leaf tips of polytrichaceous mosses, an optimal location for wind dispersal of ascospores (Figs. 2-6, 2-7) (Döbbeler 2003). *Bryophytomyces sphagni* (Navashin) Cif. (Helotiaceae, Helotiales), a pathogen of *Sphagnum* species, produces its anamorph specifically within the sporophyte capsules, replacing the moss spores and exploiting the explosive dispersal mechanism by which *Sphagnum* disperses its sexual propagules (Eckblad 1975, Chau 1979, Redhead and Spicer 1981), indicating some dispersal mechanisms among bryophilous pathogens are highly evolved.

Unlike mechanisms of fungal pathogenesis among vascular plants, little is known about the factors influencing the virulence of pathogens in mosses. Limpens et al. (2003) conducted fertilization experiments that demonstrated the virulence of *Tephrocybe palustris* attacking *Sphagnum magellanicum* Brid. and *Sphagnum papillosum* Lindb. was increased under high nitrogen conditions, and decreased under high phosphorous conditions. Similarly, the inner bands of Antarctic 'fairy rings' contain very little nitrogen in comparison to uninfected moss beds. The suggestion has been made that the rate of expansion of the pathogen may be correlated to available nitrogen (Wilson 1951). These findings indicate that nutrition could have a major impact on pathogenic moss-fungus interactions. More research is needed to determine the importance of nitrogen to other pathogenic species, and whether other macro- or microelements are important to pathogenicity. In addition, other external factors, including temperature, humidity, and the presence of other fungi, need to be examined. *Tephrocybe palustris* will destroy all available *Sphagnum* plants in axenic culture; however, in nature the pathogen is only found in small isolated patches (Untiedt and Muller 1985). Moribund and dead patches created in Antarctic mats of *Drepanocladus uncinatus* (Hedw.) Warnst. by an unidentified oomycetous species of *Pythium* can act as microniches, allowing for the establishment of higher plants, other mosses, and algae that would otherwise be unable to successfully compete, thereby altering local patterns of plant community composition (Hoshino et al. 2001). Research into abiotic and biotic parameters affecting pathogen virulence may shed light on factors controlling the balance of healthy and diseased moss

populations, and may indicate the relative importance of disease in population dynamics of bryophyte dominated areas.

Host responses to bryopathogens have been poorly characterized and are not well understood. Untiedt and Muller (1985) report that *Sphagnum fallax* chlorophyllous leaf cells infected by *Tephrocybe palustris* will form thin, partitioning, secondary cell walls. However, it is not known how these form, whether they are part of a defence mechanism, or if they arise because of irregular cell division within the leaf. The failure of crosswall formation to slow or halt the *in vitro* infection of *S. fallax* by this fungus (Redhead 1981), suggests crosswall formation is an ineffective defense mechanism. However, this response may represent evolution in action, with the host plant in the process of evolving mechanisms to halt the pathogen's advance. An unidentified ascomycetous pathogen infecting the apices of *Drepanocladus uncinatus* (Hedw.) Warnst., causes radial infection patterns in Antarctic moss mats (Longton 1973). Infected stems exhibit protoplast breakdown near the shoot apices, while a brown, necrotic zone develops lower on the stem, causing the apex to break off. The center of such ring systems recovers as the fungal infection radiates outwards in the moss mat, and healthy, lateral shoots are produced as subapical innovations from the regions below the necrotic zone on infected stems. It is unclear whether the necrotic zone on the gametophyte is a hypersensitive 'controlled suicide' response, or is caused by fungal cytotoxins. Other ascomycetes and basidiomycetes cause similar concentric infection patterns in polar moss mats, only some of which recover (Wilson 1951, Hawksworth 1973, Longton 1973, Fenton 1983). In *Physcomitrella patens* (Hedw.) Bruch., a family of genes related to the TIR-NBS class of

plant resistance genes (R-genes) of vascular plants has been identified, but their expression and functionality within the plant have not yet been characterized (Akita and Valkonen 2002). A burst of hydrogen peroxide-producing peroxidase activity may be involved in repelling attacks by fungal pathogens in *Atrichum androgynum* (Müll.Hal.) A.Jaeger; however, while the plant can produce such reactive oxygen species, their functional role in host response to infection by pathogens has not been investigated in bryophytes (Mayaba et al. 2002).

Parasitic Interactions

Host specificity varies among moss parasites and may reflect aspects of their evolutionary history. For example, the perithecial ascomycetes *Epibryon interlamellare* Döbbeler and *Bryorella cryptocarpa* Döbbeler occur solely in the interlamellar spaces of the leaves of species in the Polytrichaceae (Fig. 2-8) (Felix 1988, Döbbeler 2002). The host range of these fungi has likely been restricted by their evolution as specialists exploiting interlamellar spaces as habitats, a microniche that is present in all polytrichaceous mosses. Even narrower host ranges exist, with species such as the hyphomycete *Stemphylium botryosum* Sacc. (Pleosporaceae, Pleosporales) and the apothecial ascomycete *Helotium schimperi* Navashin (Helotiaceae, Helotiales) restricted to the single moss species, *Leptodictyum riparium* (Hedw.) Warnst. (Prior 1966) and *Sphagnum squarrosum* Crome (Redhead and Spicer 1981) respectively. This apparent host specificity could be a product of co-evolution between plant and parasite whereby these fungi are particularly well adapted to infect their host, or the product of a recent

shift to the parasitic habit and insufficient time to adapt to infect other hosts. Conversely, many moss parasites, including species of *Octospora* (Felix 1988), *Lamprospora* (Benkert 1987), *Rickenella,* and *Galerina* (Kost 1988), have broad host spectra. For example, *Octospora similis* (Kirchstein) Benkert, infects multiple species in *Bryum* (Benkert 1996), and the heterobasidiomycete *Eocronartium muscicola* (Pers.) Fitzp. (Auriculariales) infects mosses from 11 different families in the Bryales (Boehm and McLaughlin 1988). However, the question still remains whether broad host ranges indicate these fungal species have evolved as non-obligate, opportunistic parasites, or as generalists that can attack multiple species and whether a broad host range reflects a more recent evolutionary switch to a moss host.

Tissue specificity may also provide insight into the evolution and ecology in bryoparasitic fungi. For example, the basidiomycetes *Eocronartium muscicola,* and unidentified species of *Jola*, and *Rimbachia* (Fitzpatrick 1918, Boehm and McLaughlin 1988, Senn-Irlet and Moreau 2003) all produce fruiting bodies specifically on the shoot apices of host plants; it is unclear whether fruiting in this location is an adaptation that facilitates spore dispersal, or the result of competitive exclusion by fungi colonizing more proximal regions of the gametophyte. The discomycetes *Octospora* (Döbbeler 1979b, Senn-Irlet 1988, Benkert 1993) and *Lamprospora* (Döbbeler 1979b, Schumacher 1986, Benkert 1993) colonize moss rhizoids, forming appressorium-haustorium infection complexes. Again, it is not understood whether the observed tissue specificity is because of competitive restraints on the advance of the fungus, or whether abiotic environmental factors are only suitable for fungal growth within rhizoids. Similarly, ascomycetes such

as *Epibryon interlamellare* (Döbbeler 2002), and *Potriphila navicularis* (Döbbeler 1996a) fruit solely in the interlamellar spaces of polytrichaceous mosses, a phenomenon which may be explained by either spatial restrictions that result from intense competition, or exploitation of environmentally favourable micro-niches. A hitherto undescribed species of *Cladophialophora* (Davey and Currah 2007) and species of the ascomycete genera *Epibryon* (Döbbeler 1985), *Potridiscus* (Döbbeler and Triebel 2000) and *Bryonectria* (Döbbeler 1999) grow preferentially in the humid, temperature moderated greenhouse-like spaces created by the involute, overlapping lamina of *Polytrichum juniperinum,* suggesting they have specific abiotic microniche requirements. The basidiomycetes *Galerina paludosa* (Fr.) Kuhner (Redhead 1981) and *Rickenella fibula* (Bull.) Raithelh. (Kost 1988) and the apothecial ascomycete *Octospora libussae* Svrcek & Kubicka (Döbbeler and Itzerott 1981) initially colonize the filamentous juvenile tissues produced by germinating moss spores, a tactic which could be attributed either to a pioneering life-history strategy or the necessity to attack the young gametophytes before they develop resistance to the fungus. Finally, *Helotium schimperi* is specific to the mucilaginous cells found at the tips of trichome-like structures on the stems of *Sphagnum squarrosum*, suggesting a specialized parasitic relationship (Redhead and Spicer 1981).

Hyphae of fungal pathogens of bryophytes are predominantly intracellular; however, the mechanisms by which host penetration is achieved are not well understood for most species. A large proportion of fungi parasitizing mosses form appressoria that facilitate host cell penetration using mechanical pressure; however, there is extensive

diversity in their structure. The discomycetes *Octospora wrightii* (Berk. & Curt. In Berk. & Br.) J. Moravec (Benkert 1998), *Octospora musci-muralis* Graddon (Döbbeler 1979b) and *Neottiella aphanodictyon* (Kobayasi) Dissing, Korf, and Sivertsen (Benkert 1993) form spherical or elliptical dome-shaped, sometimes beaked, appressoria that force hyphal penetration pegs through rhizoid cell walls. *Discinella schimperi* forms a highly branched, cap-like appressorium over the apical portion of mucilage cells on the stem of *Sphagnum squarrosum* (Redhead and Spicer 1981). Phylogenetic analysis of appressoria-forming species may reveal evolutionary relationships that correspond with the distribution of these appressorial characters among these fungi, giving insight into their evolutionary history. The ascomycete *Potriphila navicularis* (Döbbeler 1996a), and the basidiomycetes *Eocronartium muscicola* (Fitzpatrick 1918), *Galerina paludosa* (Redhead 1981) and *Rickenella fibula* (Kost 1988) do not form appressoria and instead rely on direct perforation of the cell wall by hyphae. The fungus likely facilitates host penetration via the secretion of lytic enzymes that dissolve the cell wall (Fitzpatrick 1918). Furthermore, cell wall degradation has not been observed at locations other than penetration points (Fitzpatrick 1918, Boehm and McLaughlin 1988, Kost 1988, Döbbeler 1996a) indicating that site recognition and the penetration process are tightly controlled.

The way in which nutrients are absorbed from the host also differs among parasitic taxa. On the most basic level, the hyphomycete *Stemphylium botryosum* (Prior 1966) and the ascomycetes *Epibryon bryophilum* (Fuckel.) Döbbeler (Pseudoperisporiaceae, Dothidiomycetidae) and *Epibryon casaresi* (Bubák & Gonz. Frag.) Döbbeler (Pseudoperisporiaceae, Dothideomycetidae) (Henderson 1972) form no

specialized absorptive structures and unmodified hyphae simply grow intracellularly within the host. *Galerina paludosa* forms simple, peg-like, absorptive haustoria within the cells of the thalloid protonemata and rhizoids of *Sphagnum capillaceum* (Weiss) Schrank (Redhead 1981), while species of another bryoparasitic agaric, *Chromocyphella,* occasionally form swollen vesicles in the leaf cells, which may act as haustoria (Kost 1988). Others form more complex haustoria; *Potriphila navicularis* invades the leaf lamellae of *Polytrichum alpinum* (Hedw.) Smith and forms coiled haustoria that occupy the entire cell (Döbbeler 1996a), *Discinella schimperi* produces highly branched haustoria just within the host cell wall of mucilaginous cells on stems of *Sphagnum squarrosum* (Redhead and Spicer 1981) and *Leptomeliola mnii* Racov. (Parodiopsidaceae) forms flabelliform haustoria within the rhizoids of *Mnium punctatum* (Racovitza 1959). Similarly, the apothecial ascomycete *Gloeopeziza cuneiformis* Döbbeler (Helotiaceae, Helotiales) forms highly branched, coiled haustoria that occupy cells of the leaf lamellae in species of *Polytrichum* (Döbbeler 1996b). Finally, *Eocronartium muscicola* is a systemic parasite, growing both inter- and intracellularly. Its basidiocarp eventually replaces the sporophyte, allowing the fungus to commandeer the transfer cells that would otherwise supply nutrients to the developing sporophyte's foot, seta, capsule, and spores (Fitzpatrick 1918, Boehm and McLaughlin 1988). Other fungi including *Jola javensis* Pat. (Boehm and McLaughlin 1988), *Rimbachia bryophila* (Pers.) Redhead, *R. arachnoidea* (Peck) Redhead, *R. neckerae* (Fr.) Redhead (Senn-Irlet and Moreau 2003), and *Bryostroma rhacomitrii* Döbbeler (Döbbeler 1978), produce sexual fruiting bodies at the apices of the moss gametophytes they infect, often entirely

replacing the stem tip; however it is currently unknown if these fungi employ nutrient-obtaining tactics similar to *Eocronartium muscicola*. Although the basic structure of different bryoparasitic haustoria has been well described, there are still unanswered questions as to whether a lack of specialized absorptive structures might correlate with a recent shift to the bryoparasitic habit, and whether different haustorial structures are more efficient in facilitating nutrient absorption from the host.

Infection by fungal parasites elicits a variety of symptoms among host mosses. In the sporodochial ascomycete *Schizotrichella lunata* Morris (Bowen 1968) and species of the basidiomycete *Rimbachia* (Senn-Irlet and Moreau 2003), infection causes only occasional mild chlorosis. More obvious responses to fungal infection are evident in species of *Mnium*, *Polytrichum*, and *Drepanocladus* attacked by *Arrhenia retiruga*, where thickening and browning of host cell walls occur around invading intercellular hyphae (Kost 1988). Similarly, *Mnium punctatum* infected by *Leptomeliola mnii* forms melanized papilla-like structures at penetration sites (Racovitza 1959). It is unclear if these cell wall modifications are a defensive response, or a deformation caused by the fungus. *Stemphylium botryosum* grows intracellularly through the gametophyte of *Leptodictyum riparium* and forms conidiophores bearing single conidia in the leaf axils. Infected plants are stunted, and leaves lack costae (Prior 1966), indicating interference with the normal developmental patterns. However, the manner by which infection with *S. botryosum* induces these changes in the host has not been investigated. Rhizoid parasites in *Octospora* can cause the formation of single-celled, hypertrophic galls in the

meristematic regions of rhizoids (Fig. 2-9)(Döbbeler 1979b, Benkert 1998, Senn-Irlet 1988, Döbbeler 1997), but the mechanism of gall induction is unknown.

The ecology of bryoparasitic relationships has received little attention. Most bryopathogenic fungi have not been classified as obligate or non-obligate parasites, and the manner in which factors such as temperature, moisture, and host health impact the infectivity of bryoparasites has not been investigated. Furthermore, the extent to which these associations are dynamic and dependent on abiotic and biotic factors is unkown. *In vitro*, moss protonemata can act as platforms for lichen formation, with the fungal component growing parasitically until appropriate algal symbionts are encountered, upon which the parasitic relationship is terminated (Bonnier 1888, 1889). However, it has not been investigated whether fungi can exist endophytically as latent parasites that do not manifest until specific host or environmental conditions are present, and whether opportunistic parasitism of damaged or stressed bryophytes occurs.

Saprobic Interactions

The cell walls of mosses decompose slowly, ostensibly because they contain polyphenolic-rich, lignin-like compounds that are either unsuitable as a substrate or toxic to the majority of microorganisms (Verhoeven and Liefveld 1997). However, some microfungi are capable of degrading these compounds and appear to play a significant role in bryophyte decomposition. Loculoascomycetes, including members of the Mycosphaerellaceae, have been reported as saprobes on dead sporophytes of various species (Racovitza 1959).

Thormann et al. (2002) conducted carbon substrate utilization analyses on microfungi isolated from *Sphagnum fuscum* and concluded that a diverse suite of fungi producing a variety of extracellular enzymes including amylases, cellulases, and polyphenoloxidases were capable of collectively degrading the cell wall components of the dead plants. Research by Tsuneda et al. (2001c) and Rice et al. (2006) demonstrates that different peat-inhabiting microfungi including *Oidiodendron maius* Barron, *O. periconioides* Morrall (Myxotrichaceae), and *Pochonia bulbillosa* (W. Gams & Malla) Zare & W. Gams (Hypocreales) have distinct and unique methods of degrading the cell walls of *Sphagnum fuscum* (Fig. 2-10, 2-11).

Interactions Involving Mycorrhizal Fungi

Fungi known to form mycorrhizal associations with gymnosperms and angiosperms have also been found to form structurally analogous mycorrhiza-like associations with lower land plants, including hepatophytes, lycopods, and ferns (Read et al. 2000). Although mosses may also be colonized by fungi known to form mycorrhizal associations with higher plants, and some structures characteristic of these associations may be formed, there is no experimental evidence for the transfer of photoassimilate across a living interface to the fungus or nitrogen and phosphorous to the plant. Consequently, it is generally thought that while moss gametophytes provide a favourable environment for the growth of mycorrhizal fungi, they are not suitable mycorrhizal partners (During and van Tooren 1990). Instead, associations between mosses and mycorrhizal fungi associated with other plants appear to be primarily saprobic.

Mycorrhizal fungi are known to utilize mosses as a source of nutrients and appear to exploit the plants in two ways. Alternate periods of wetting and drying result in the release of nutrient-rich aqueous leachates from moss gametophytes, which are used by mycorrhizal fungi that proliferate in the surface soil layers (Carleton and Read 1991). Many vesicular-arbuscular and ectomycorrhizal fungi also colonize the dead and senescent portions of mosses (Rabatin 1980, Berch and Fortin 1983, Warner 1984, Carleton and Read 1991). Carleton and Read (1991) used radioactive tracing experiments to demonstrate the transfer of phosphate and carbon from *Pleurozium schreberi* (Brid.) Mitt. to *Pinus contorta* Dougl. via the ectomycorrhizal *Suillus bovinus* (Pers.) Kuntze (Suillaceae, Boletales), whose mycelium had invaded moribund portions of the gametophytes. Mycorrhizal fungi colonizing dead and senescent bryophyte matter likely do not simply compete for nutrients liberated by other decomposer fungi, but actively attack the substrate and enzymatically mobilize both nitrogen and phosphorous (Bending and Read 1995). Furthermore, it appears that mycorrhizal fungi can 'defend' these nutrient sources, as once a nutrient-rich portion of litter is colonized by a mycorrhizal fungus, further decomposition by soil bacteria is prevented (Ponge 1990).

Mycorrhizal fungi have developed a variety of structures to facilitate the absorption and transfer of nutrients. Arbuscular mycorrhizal (AM) fungi, including *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe and *G. caledonium* (Nicolson & Gerd.) Trappe & Gerd., form rudimentary appressoria that mechanically facilitate their entry into the cells of dead and senescent mosses (Warner 1984, Iqbal and Bareen 1990). In species where appressoria are absent, it is unknown whether the fungi obtain entry into

cells through enzyme digestion of the cell wall or through opportunistic utilization of wounds and damaged or moribund cells. Intracellular structures formed in mosses by invading mycorrhizal fungi are very diverse in form. *Endogone pisiformis* Berk. forms highly branched structures *in vitro* (Berch and Fortin 1983), while AM fungi form a variety of vesicular structures that vary in size, shape, septation, and wall structure (Parke and Linderman 1980, Iqbal et al. 1988, Iqbal and Bareen 1990, Jakucs et al. 2003). It has yet to be determined whether these structures facilitate absorption of nutrients from the moss substrate or form simply as a host recognition response by the fungus when it contacts any type of plant, including mosses. Furthermore, Jakucs et al. (2003) suggest that the absence of arbuscules in mosses colonized by AM fungi may be attributable to seasonal fluxes in nutrient availability or the ephemeral nature of arbuscules. Detailed characterization of the host-fungus interface in these associations is needed to verify whether arbuscule formation is related to nutrient availability and in what manner nutrient transfer is occurring between the host and the invading mycorrhizal fungus.

The ecological relevance of moss-mycorrhizal fungi associations, beyond saprobic interactions, is not well understood. *In vitro* research suggests that hyphal fragments and vegetative spores of *Glomus mosseae* and *G. caledonium* retain their mycorrhizal infective ability for only 48 hours in the absence of host roots, while hyphal fragments within roots or litter have survival times of months (Warner 1984). It appears that dead and senescent mosses that have been colonized by mycorrhizal fungi can act as an inoculum reservoir that ensures colonization of newly growing roots in the spring by mycelium that grows from living hyphae within the mosses (Rabatin 1980, Warner 1984,

Iqbal and Bareen 1990). Further research is needed to determine the importance of this inoculum reservoir *in vivo* in relation to the presence of fungal resting spores in the soil that are also capable of causing infection.

Other Interactions

Mosses and fungi have also been reported to engage in a number of commensal interactions. For example, Hahn and Bopp (1972) describe an unknown substance being produced by the fungi *Aspergillus niger* Tiegh., *Alternaria solani* (Ellis & Martin) Jones & Grout, and an unidentified species of *Alternaria* that promoted protonematal growth and bud formation in *Funaria hygrometrica* Hedw.. However, there is no information on the identity of the active substance or its mode of action within the moss. Similarly, the presence of fungi isolated from *Brachythecium rutabulum* (Hedw.) B.S.G. allows this plant to grow over a much wider pH range in culture than when the fungi were absent (During and van Tooren 1990). It is unclear whether this phenomenon is attributable to the fungus modifying the substrate and buffering extremes in pH, or growing endophytically within the moss and producing a substance that increases pH tolerance of both parties.

Bryophytes have also been reported to produce compounds with fungistatic and fungitoxic properties against a variety of basidiomycetes, ascomycetes, and hyphomycetes (Wolters 1964, van Hoof et al. 1981). *Plagiothecium denticulatum* (Hedw.) Schimp. is of particular interest, as extracts of the plant significantly inhibit growth of common hyphomycete pathogens such as *Rhizoctonia solani*, *Fusarium*

bulbigenum and *Botrytis allii* (Wolters 1964). However, the active fungistatic and fungitoxic compounds in mosses such as *Plagiothecium denticulatum* have yet to be identified and may provide new sources of anti-fungal compounds.

Like tracheophytes, bryophytes harbour endophytic fungi that have no apparent detrimental effects on their hosts (Thormann et al. 2001, Jakucs et al. 2003). Endophytic fungi in the roots of vascular plants, including *Heteroconium chaetospira* (Grove) Ellis, may imbue their host plants with resistance to pathogens including *Verticillium dahliae* Kleb. and *Plasmodiophora brassicae* Wor. (Narisawa et al. 1998, Narisawa et al. 2002). Other endophytic fungi have been reported to act as latent pathogens or parasites, remaining dormant and benign until the plant is under stress and susceptible to attack (Shearer 2002). The functional role of fungal endophytes in bryophyte ecology has yet to be investigated, and questions remain as to whether they have comparable roles to those endophytes found in vascular plants, and whether common endophytic species of vascular plants, such as the root endophyte *Phialocephala fortinii* Wang & Wilcox (Wang and Wilcox 1985), have host ranges that encompass bryophyte taxa as well.

A variety of fungi have also been observed to grow directly on mosses without penetrating the moss cells and causing visible symptoms. For example, *Epibryon interlamellare* appears to be ubiquitous on *Polytrichum juniperinum* (Fig. 2-8); however, it has not been observed to penetrate the host or degrade it using extracellular enzymes, suggesting the fungus might be deriving its nutrition from leachates released during wetting and drying cycles (Carleton and Read 1991). Similarly, *Melanopsamma interlamellaris* Racov. (Chaetosphaeriaceae, Sordariales) grows between the leaf lamellae

of polytrichaceous mosses without penetrating cells and appears to use the bryophyte only as a supporting physical substrate (Racovitza 1959).

Further Research

In vivo observations of bryophilous fungi have alluded to unique functional roles for this group; however, the physiology and etiology of these interactions is still poorly understood. The next step in the study of bryophyte-fungus interactions is the *in vitro* culturing of bryophilous fungi and the inoculation of these fungi onto axenically grown moss to create controlled experimental systems for detailed physiological, anatomical, biochemical, and genetic studies. Many moss species, including *Aulacomnium palustre* (Hedw.) Schwaegr., *Hylocomium splendens* (Hedw.) Schimp., *Dicranum polysetum* Sw., *Pleurozium schreberi* (Brid.) Mitt., *Pylaisiella polyantha* (Hedw.) B.S.G., *Funaria hygrometrica* Hedw., and *Tetraplodon mnioides* (Hedw.) B.S.G., can be established in axenic culture from spores and will grow readily on White's media (Loveland 1956) solidified with Phytagel™ (Sigma) under a 12 hour alternating light-dark regime at 75% relative humidity. Axenically grown moss can then be inoculated with fungal spore suspensions or agar plugs to create *in vitro* model systems for the study of moss-fungus interactions.

In *vitro* resynthesis of bryopathogenic-fungus relationships will allow for the determination of pathogen enzyme and toxin production, mechanisms of host cell disruption, anamorph-teleomorph connections, the nature of host responses to infection, virulence factors, and the genetic elements controlling these characters, providing insight

into the life history, etiology, ecology, evolutionary history, and physiology of bryophilous pathogens. Similar experiments with bryoparasitic fungi will allow for investigation of the role of competition and abiotic factors in determining microniches within the moss gametophyte and the etiology, anatomy, and physiology of the host-parasite relationship. Further studies of saprobic and mycorrhizal fungi cultured *in vitro* are needed to elucidate mechanisms of bryophyte cell wall degradation and provide insight into nutrient cycling in moss rich ecosystems. In order to fully understand the nature of fungus-bryophyte interactions it is imperative that the fungal and plant members of the association are cultured *in vitro* to allow for detailed physiological, biological, genetic, and ecological experimentation.

Figures 2-1 to 2-5.
Scleroconidioma sphagnicola* on *Sphagnum fuscum.

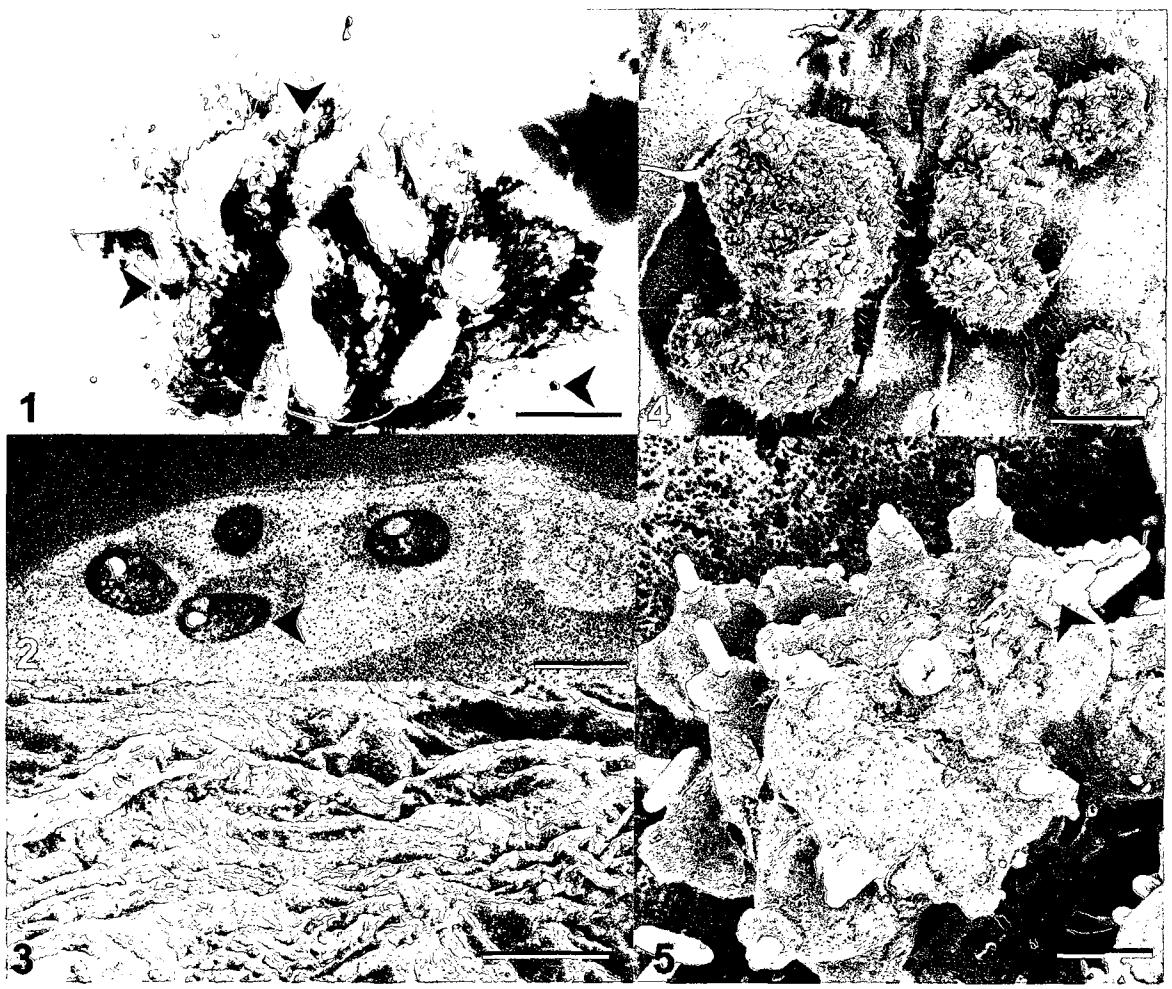
Figure 2-1. Capitulum of *Sphagnum fuscum* infected by *Scleroconidioma sphagnicola.* *Sphagnum* leaves are exhibiting both chlorosis and necrosis, and fungus is producing microsclerotia on the plant's surface (arrowheads). Scale bar = 400 μm (Adapted from Tsuneda et al. 2001a)

Figure 2-2. Transmission electron micrograph of a *Sphagnum fuscum* cell showing pathogenic hyphae growing inside the cell wall and creating localized voids around themselves (arrowheads).

Figure 2-3. Scanning electron micrograph of the surface of *Sphagnum fuscum* leaf cells infected with *S. sphagnicola* showing wavy deformations of cell walls. Scale bar = 4 μm. (Adapted from Tsuneda et al. 2001a)

Figure 2-4. Microsclerotia of *Scleroconidioma sphagnicola* that have been converted into conidiomata. Scale bar = 100 μm. (Adapted from Tsuneda et al. 2000)

Figure 2-5. Enlargement of Fig. 2.3 showing conidiogenous cells on the surface of microsclerotia producing conidia (arrowheads). Scale bar = 2 μm. (Adapted from Tsuneda et al. 2000)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Figures 2-6 to 2-11.

Fungal parasites and saprobes of bryophytes.

Figure 2-6. Abaxial side of *Polytrichum juniperinum* leaf showing perithecia (arrowheads) of a *Bryochiton* species in various stages of development. Scale bar = 350 μm.

Figure 2-7. Abaxial side of *Polytrichum juniperinum* leaf showing network of short, melanized hyphae and a developing perithecium of *Bryochiton*. Scale bar = 50 μm.

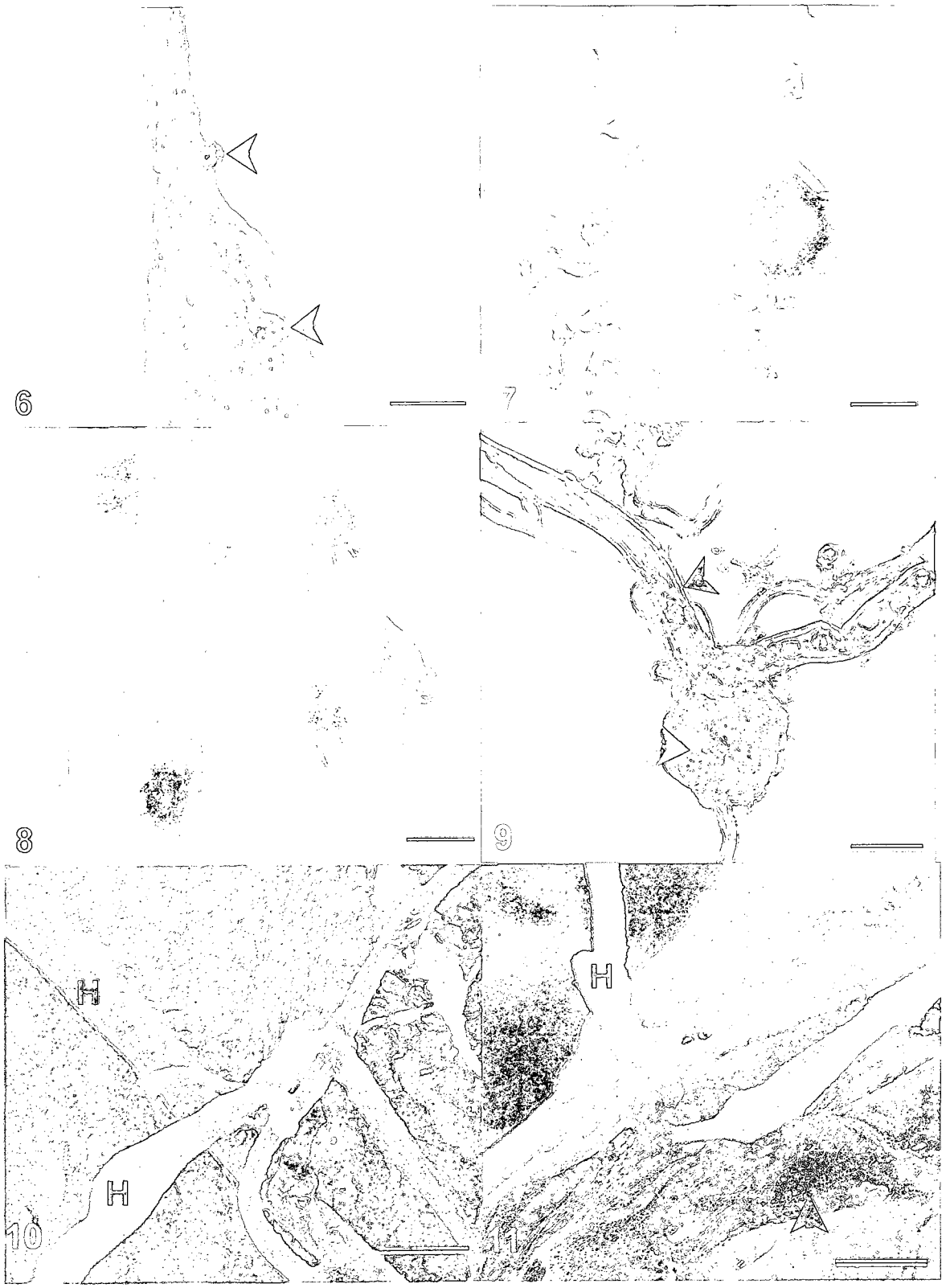
Figure 2-8. Adaxial view of *Polytrichum* leaf infected with *Epibryon interlamellare*. Note the setose perithecia form between the leaf lamellae but do not appear to interfere with host or cause obvious symptoms of chlorosis and necrosis. Scale bar = 40 μm. (B. Senn-Irlett, Personal Communication 2005).

Figure 2-9. *Octospora orthotricha* infecting *Orthotrichum diaphanum*. The fungus has caused the formation of a gall (white arrowhead) on the bryophyte's rhizoid (black arrowhead). Scale bar = 20 μm. (B. Senn-Irlett, Personal Communication 2005).

Figure 2-10. Selective degradation of the outer cell wall of *Sphagnum fuscum* by *Pochonia bulbillosa*. Fungal hyphae (H) grow both outside and within cell wall, creating

wavy deformations of the wall (arrowhead). Scale bar = 4 μm. (Adapted from Tsuneda et al. 2001c).

Figure 2-11. Simultaneous degradation of cell wall components of *Sphagnum fuscum* by hyphae (H) of *Oidiodendron maius* creating localized voids in cell wall (arrowhead). Scale bar = 7 μm. (Adapted from Tsuneda et al. 2001c).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Chapter 3

Cryptic ascomycetous associates of bryophytes.

Introduction

The microbial community associated with mosses (Bryophyta) is not well known (Davey and Currah 2006), although surveys of ascomycete and basidiomycete fruiting bodies (Racovitza 1959, Felix 1988, Kost 1988, Döbbeler 1997) suggest a taxonomically broad array of fungi form associations with these hosts. Culture based investigations of the species richness associated with bryophytes has been limited to specific moss-dominated communities such as Antarctic tundra (Kerry 1990, Möller and Dreyfuss 1996, McRae and Seppelt 1999, Tosi et al. 2002) and peat-forming wetlands (Thormann et al. 2001). These studies have detected additional species diversity among those fungi that colonize mosses without forming ostentatious sporulating structures. This suggests culture-based approaches are needed to make a true approximation of the biodiversity of bryophilous fungi.

Both the shoots (Arnold and Lutzoni 2007, Arnold et al. 2007) and roots (Vandenkoornhuyse 2002, Addy et al. 2005) of vascular plants are known to play host to cryptic, endophytic associates that represent at least four fungal phyla. Recent studies indicate that non-vascular plants, including hepatophytes (Davis and Shaw 2008) and bryophytes (Thormann et al. 2001, Kauserud et al. 2008), are also host to cryptic endophytic fungal communities, although they have not been characterized as thoroughly as those found in vascular plants and likely represent a source of undescribed biodiversity among bryophilous fungi. In vascular plants, fungal endophytes have been implicated as

latent pathogens and parasites, as agents conferring anti-herbivory and disease resistance to their hosts, and as commensal, asymptomatic inhabitants of the plant tissues, and are transmitted both vertically and horizontally. Given the diversity of function observed among vascular plant endophytes, further characterization of those cryptic fungal inhabitants of mosses would be expected to elucidate similar functional roles for endophytes of bryophytes.

During a survey of cultureable bryophilous fungi inhabiting forest mosses in Canada, a number of cryptic fungi were isolated from surface sterilized moss gametophytes. For the purpose of this study those fungi isolated in this manner are considered to be endophytes *sensu lato* (fungi actively growing within host tissues), on the premise that the host surface sterilization eliminated casual phylloplane fungi that are simply present on the surface, and not actively associating with its tissues. Phylogenetic analysis of the internal transcribed spacer (ITS) region of ribosomal DNA was used to determine if these endophytic isolates represent previously unknown taxonomic diversity among bryophilous fungi, and if they display affinities to fungal groups to which specific ecological functions have been ascribed.

Materials and Methods

Collections of gametophytes of nine moss host species [*Aulacomnium palustre* (Hedw.) Schwaegr., *Hylocomium splendens* (Hedw.) B.S.G., *Polytrichastrum alpinum* (Hedw.) G.L. Smith, *Polytrichum juniperinum* (Hedw.), *Pleurozium schreberi* (Brid.) Mitt., *Pylaisiella polyantha* (Hedw.) B.S.G., *Sphagnum fuscum* (Schimp.) Kling.,

Sphagnum squarrosum (Crome), *Brachythecium velutinum* (Hedw.) B.S.G., and *Plagiomnium cuspidatum* (Hedw.) Koponen] were made at eleven locales in Canada (Table 3-1). Gametophytes were rinsed under running distilled water and examined with a stereomicroscope to locate fungal fruiting bodies and spore masses on the gametophyte surfaces, which were identified by morphological characters if possible. Gametophytes were subsequently surface sterilized, fragmented, and incubated in moist chambers or on isolation media [oatmeal agar (OA): 20 g/L ground oatmeal, 20 g/L agar (Invitrogen, Carlsbad, CA); mycosel (MYC): 36 g/L Mycosel Agar (Becton, Dickinson & Co., Sparks, MD); potato dextrose agar (PDA): 39 g/L potato dextrose agar (Becton, Dickinson & Co. Sparks, MD); mineral agar (MIN): 4.4 g/L K_2HPO_4, 1.7 g/L KH_2PO_4, 2.1 g/L NH_4Cl, 0.195 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, 0.05 g/L $FeSO_4$, 0.003 g/L $CaCl_2$, 15 g/L agar] amended with 0.01% oxytetracycline (Sigma, St. Louis, MO) as described by Davey and Currah (2007). Emerging hyphae were subcultured onto OA or PDA and sporulating cultures were identified morphologically.

DNA fingerprinting techniques were used to generate molecular characters from the internal transcribed spacer region (ITS) of the rDNA for thirty one sterile or morphologically ambiguous isolates. Genomic DNA of each was extracted by CTAB extraction (Davey and Currah 2007), and purified using a QIAquick PCR Purification kit (Qiagen, Mississauga ON, Canada). The ITS region was amplified using the forward and reverse primer sets ITS 1 and ITS 4 (White et al. 1990) or BMB-CR (Lane et al. 1985) and ITS 4 (White et al. 1990) and amplicons were purified as described by Davey and Currah (2007). Purified amplicons were sequenced with an ABI 3100 automated

sequencer (Amersham Pharmacia Biotech, Piscataway, NJ) and the primers ITS 1, ITS 2, ITS 3, ITS4, (White et al. 1990) and BMB-CR (Lane et al. 1985). BLAST (Altschul et al. 1990) comparisons were used to place the 31 isolates at the class or sub-class level and data matrices of ITS sequences from the Chaetothyriomycetidae (Eurotiomycetes), Dothideomycetes, Hypocreomycetidae (Sordariomycetes), Leotiomycetes, Sordariomycetidae (Sordariomycetes), and Xylariomycetidae (Sordariomycetes) were subsequently created from sequences retrieved from GenBank. Sequences of each matrix were aligned using MAFFT v5.8 (Katoh et al. 2005) and the subsequent alignment was manually verified. These matrices were subjected to maximum parsimony analysis using PAUP v.4.0b10 (Swofford 2003) with Fitch parsimony, random simple step-wise addition of taxa, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Support for branching topologies was evaluated using 1000 resamplings of the data by bootstrapping analysis using the same criterion described above (Felsenstein 1985). All trees were scored for length in steps, consistency index (CI), retention index (RI), and homoplasy index (HI). Family-level sub-alignments were created with additional sequences retrieved from GenBank and subjected to parsimony analysis and bootstrapping as described above to confirm generic- and species- level affinities (data not shown).

Results

A total of 251 isolates were observed or isolated, and identified from the moss gametophytes (Tables 3-2, 3-3). Of these, 93% belonged to the Ascomycota, and only

7% of isolates recovered belonged to the Basidiomycota and Zygomycota. These isolates were discarded as non-target individuals, and the remaining isolates were considered for further investigation.

A group of 31 sterile or morphologically ambiguous isolates was sequenced, and its members displayed phylogenetic affinities to eight orders that were distributed among the ascomycete classes Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes (Table 3-2). Six isolates were allied with members of the Chaetothyriales (Chaetothyriomycetidae, Eurotiomycetes) (100%) (Fig. 3-1), four of which grouped with the genus *Rhinocladiella* with high bootstrap support (99%), and two of which grouped with species of *Cladophialophora*. Three isolates displayed phylogenetic affinities to members of the Pleosporales (Dothideomycetes) (92%), including *Dendryphion nanum* and *Atradidymella muscivora* (Fig. 3-2). Three isolates were allied with the Helotiales (Leotiomycetes) (Fig. 3-3), of which one was moderately supported (84% bootstrap support) as part of the genus *Hymenoscyphus*, another was allied to the genus *Cadophora*, while the third was contained within a well supported (100%) clade representing the Sclerotiniaceae. Eighteen of the thirty-one isolates had phylogenetic affinities to the Sordariomycetes. Among the Xylariales (Xylariomycetidae, Sordariomycetes), two isolates grouped with *Nemania serpens* with high bootstrap support (100%) while another isolate was allied to the genus *Kretzschmaria* (100% BS support) (Fig. 3-4). A single isolate was nested within the Sordariales (Sordariomycetidae, Sordariomycetes), forming a strongly supported clade (100%) with the genus *Cercophora*. Four isolates displayed phylogenetic affinities to the genus

Coniochaeta in the Coniochaetales (Sordariomycetidae, Sordariomycetes) (88%) (Fig. 3-5). Within the Hypocreomycetidae (Sordariomycetes), ten isolates displayed phylogenetic affinities to the Hypocreales (Fig. 3-6). An isolate from *Hylocomium splendens* was strongly supported as part of the genus *Cosmospora* (94%), while two other isolates displayed affinities for *Nectria.* Three isolates were closely allied to species of *Fusarium*, while an additional four isolates nested within the strongly supported *Hypocrea/Trichoderma* clade. Only five of the thirty one isolates demonstrated phylogenetic affinities to taxa that have previously been reported from moss substrates.

An additional 56 species distributed among 12 orders in the Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes were identified from sporulating cultures, and spores and fruiting bodies found during stereomicroscope scans (Table 3-3). These fungi included common cosmopolitan taxa, saprobic taxa and taxonomically diverse suites of fungi that are known bryophyte-specific associates, and known pathogens of vascular plants. Only six of the 56 species were also detected among the cryptic, non-sporulating isolates. Among the species observed, 27 represent new reports of associations with moss hosts.

In excess of 40 isolates were identified by morphological or molecular means from each of three common boreal forest bryophytes: *Polytrichum juniperinum, Hylocomium splendens,* and *Aulacomnium palustre*. Of the three mosses, *P. juniperinum* was found to have the highest species richness among its associated fungi (34 species, 12 orders) followed by *H. splendens* (25 species, 11 orders) and *A. palustre (*18 species, 7

orders). Nineteen of the 34 species identified from *P. juniperinum* were not isolated from any other bryophyte host, while 12 species were found only in association with *H. splendens*, and 4 species were found only in association with *A. palustre*.

Discussion

Molecular characterization of the 31 sterile or morphologically ambiguous isolates and morphological identification of sporulating isolates identified 21 species or operational taxonomic units (OTU's) belonging to 15 ascomycete orders, of which 13 (62%) represent new reports of associations with bryophyte hosts. The high proportion of new host reports among the fungi observed, in conjunction with their broad taxonomic diversity confirms bryophytes as an additional source of fungal biodiversity that have been historically overlooked in most ecosystems (Davey and Currah 2006). Only a small proportion of sterile isolates represented taxa that were also identified during morphological screening, indicating that morphological and molecular approaches are complementary, rather than redundant. The paucity of basidiomycete and zygomycete cultures recovered is not likely a result of an ascomycete predilection for bryophyte hosts to the exclusion of other taxonomic groups, and more likely a result of methodological culture and detection biases similar to those observed by Arnold et al. (2007) in their studies of foliar endophytes. As such, a true approximation of the biodiversity associated with bryophyte hosts could best be attained through combined morphological and molecular techniques (both using cultures and culture-free).

Among the bryophytes for which more than forty associated fungal isolates were identified, *Polytrichum juniperinum* was host to the greatest species richness. Polytrichaceous mosses have a complex leaf anatomy that includes multicellular leaf lamellae and a leaf lamina that is often involute. This complexity is thought to create more microniches and potential habitat for fungi in Polytrichaceous mosses (Döbbeler 2002) than are present in other, less anatomically complex mosses, including *H. splendens* and *A. palustre,* allowing species like *P. juniperinum* to host more diverse fungal communities.

Twenty percent of the sterile isolates associated with bryophytes analyzed in this study displayed phylogenetic affinities to members of the Chaetothyriales (Chaetothyriomycetidae, Eurotiomycetes), a group whose members have previously been reported as saprobes, pathogens, and foliar and root endophytes of vascular plants (Barr 1987, Geiser et al. 2006, Arnold et al. 2007, Narisawa et al. 2007), as well as opportunistic human pathogens (Haase et al. 1999). Four isolates (Hs5-A, Pj9-C, Psp1-K, Psp1-M) from three different host species were allied with the genus *Rhinocladiella*, whose species are frequently reported as saprobes as well as opportunistic human pathogens (de Hoog 1977, Arzanlou et al. 2007). *Rhinocladiella atrovirens* and *Ramichloridium anceps* were also identified among the sporulating isolates in this study, suggesting that mosses may represent a previously unknown reservoir of inoculum for this group of human pathogens. A single isolate from *Polytrichum juniperinum* was closely allied with *Cladophialophora minutissima*, a presumed saprobic species that is known only from bryophyte hosts, including *P. juniperinum* (Davey and Currah 2007).

Cladophialophora minutissima was also identified among sporulating isolates in this study suggesting it is a common fungal associate of bryophytes. An isolate from *Pleurozium schreberi* (Ps6-C) displayed phylogenetic affinities to *Cladophialophora chaetospira*, a herpotrichiellaceous soil saprobe (Domsch et al. 1993) and endophyte of vascular plant roots that is known to confer pathogen resistance to its hosts (Hashiba and Narisawa 2005, Narisawa et al. 2007). The presence of this fungus as an endophyte in mosses may be of dual ecological significance, as *C. chaetospira* may act similarly in bryophytes as it does in vascular plants, conferring disease resistance to its host. However, it may also be of significance in the context of horizontal endophyte transmission within the boreal forest, where seeds of vascular plants frequently germinate within moss mats and do not initially have contact with the soil and the inoculum reservoir contained therein. The moss mat itself may represent an additional inoculum reservoir for horizontal transmission of root endophytes of vascular plants.

Seven isolates (Ap1-R, Hs3-A, Plsp1-G, Ps5-H, Ap1-N, Hs7-F, Pj7-G) displayed affinities to the Pleosporales (Dothideomycetes), Capnodiales (Dothideomycetes), or Helotiales (Leotiomycetes) whose members are frequently reported as endophytes and pathogens of vascular plants, or saprobes of plant material (Schoch et al. 2006, Wang et al. 2006). In particular, isolates grouped with taxa such as *Cadophora* (Helotiales, Leotiomycetes), *Dendryphion nanum* (Pleosporales, Dothideomycetes), and *Hymenoscyphus* (Helotiales, Leotiomycetes), which are commonly known as saprophytes of vascular plant materials (Lizon 1992, Domsch et al. 1993). Given that bryophyte cell walls, like vascular plant cell walls, contain both cellulosic and polyphenolic components

(Edelmann et al. 1998) it is likely that those cell wall extracellular enzymes produced by these fungi that allow them to decompose vascular plant substrates, would also allow them to act as saprophytes on bryophyte material. Among the pleosporalean and helotialean fungi recovered were several isolates (Ap1-R, Hs7-F, Pj7-G) that demonstrated phylogenetic affinities to taxa that are frequently plant pathogenic. One isolate (Ap1-R) grouped strongly with *Atradidymella muscivora,* a pleosporalean pathogen known only from moss hosts (Davey and Currah 2008, unpublished). Species diversity among dothideomycetous pathogens of mosses appears to be particularly high, given the identification of additional isolates or occurrences of other taxa, including *Scleroconidioma sphagnicola,* and species of *Epibryon* and *Bryochiton*, among the sporulating taxa in this study. A second sterile isolate (Pj7-G) was allied with members of the Sclerotiniaceae (Helotiales), a family whose members commonly occur as pathogens of vascular plants (Whetzel 1945) and have been previously reported as pathogens of bryophytes (Racovitza 1959). Furthermore, while species of *Hymenoscyphus* are generally classified as saprobes, there are also reports of a single species, *Hymenoscyphus subcarneus,* that is a pathogen of mosses (White 1942). Further characterization of the host-fungus interface is needed to determine the ecological function of these sterile isolates with phylogenetic affinities to vascular plant pathogens and saprobes.

Among isolates with phylogenetic affinities to the Sordariomycetes were three isolates (Psp1-O, Sf4-B, Pj9-H) with affinities to members of the Xylariales (*Nemania serpens* and *Kretzschmaria*). Members of the Xylariales are frequently reported as

pathogens, endophytes, and saprobes of vascular plants, and it has been suggested that some species can alternate hosts, acting as an endophyte or pathogen on one, and a saprobe on another (Rogers 2000). In particular, *Nemania serpens* has been reported to 'switch hosts' in this fashion (Carroll 1999). Its isolation in this study suggests that bryophytes represent a previously unknown group of hosts on which the fungus can persist when one host is unavailable, and points to a new ecological function of bryophytes as alternate hosts for plant pathogens, saprobes, and endophytes. Hepatophytes have also been identified as hosts to endophytic members of the Xylariales, and may play a similar ecological role (Davis et al. 2003, Davis and Shaw 2008). However, in this study, members of the Xylariales accounted for only 9% of total species richness among those cryptic, presumably endophytic isolates recovered from bryophyte hosts, which is in stark contrast to the Xylariaceae-dominated endophytic fungal communities reported for hepatophytes (Davis et al. 2003, Davis and Shaw 2008).

Four of the isolates sequenced (Ap1-D, Ap1-K, Sf4-C, Hs6-GJ10) displayed phylogenetic affinities to the genus *Coniochaeta* (Coniochaetales, Sordariomycetidae) and additional isolates of *Lecythophora*, an anamorph of *Coniochaeta*, were identified among the sporulating isolates in this study (Fig. 3-5, Table 3-3). These records are the first report of members of the Coniochaetales from moss hosts, and *Coniochaeta* species have been hitherto known as saprobes of vascular plant material and dung, and endophytes or weak pathogens of vascular plants (Mahoney and LaFavre 1981, Checa 1988, Weber 2002). *In vitro* characterization of the interactions between a bryophilous isolate of *C. velutina,* a weak pathogen of angiosperm trees, and the moss *Funaria*

indicate the fungus also acts as a weak, opportunistic parasite, and latent saprobe of bryophytes (Davey et al. 2008, unpublished). As such, it appears unlikely that members of *Coniochaeta* are restricted in their host ranges, and are likely able to act as saprobes or pathogens on a variety of vascular and non-vascular plant hosts.

Cryptic and morphologically ambiguous isolates with affinities to the Hypocreomycetidae were primarily allied with mycoparasitic taxa, including species of *Hypocrea* (anamorph: *Trichoderma*), *Cosmospora,* and *Fusarium* (Rossman et al. 1999). Other mycoparasitic taxa, including additional species of *Trichoderma* and *Sphaerodes fimicola* (Melanosporales) were identified among sporulating isolates, suggesting the fungi associated with mosses create an additional microniche on their hosts that sustains populations of fungicolous members of the Hypocreomycetidae. In addition to known mycoparasites, several isolates displayed phylogenetic affinities to *Nectria*, a genus that is typically reported as saprophytic and a weak pathogen of woody vascular plants (Rossman et al. 1999). While bryophyte cell walls contain cellulosic and polyphenolic components similar to those found in vascular plants that may render them suitable substrates for saprophytes more frequently reported from vascular plants, species of *Nectria* have also been reported as host specific pathogens of mosses (Racovitza 1959, Döbbeler 1988). Detailed studies of the host-fungus interface of these isolates is needed to determine what ecological role they fulfill in their bryophyte hosts, and whether these isolates represent sterile, cryptic cultures of bryophilous nectriaceous species that have previously been classed as obligate biotrophs or pathogens (Racovitza 1959, Döbbeler 2002).

Analysis of fungi that can be cultured from bryophyte hosts has revealed considerable previously undescribed biodiversity among bryophilous ascomycetes and confirms the presence of a taxonomically diverse, cryptic fungal community associated with mosses. The advent of molecular techniques that allow both culture-free characterization of cryptic fungi and characterization of those fungi cultured from bryophyte hosts represents an exciting opportunity to further circumscribe the diversity of fungi associated with bryophytes. However, much more detailed studies of host-fungus interactions are needed to determine the functional ecology of these fungi in relation to their bryophyte hosts.

Figure 3-1. One of 2 equally parsimonious trees (1189 steps, CI=0.537, RI=0.633, HI=0.463) showing the placement of bryophilous fungi among the Chaetothyriomycetidae (Eurotiomycetes). Trees were inferred from maximum parsimony analysis of ITS sequences. The analysis included 567 characters, of which 215 were constant, 78 were uninformative, and 274 were parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

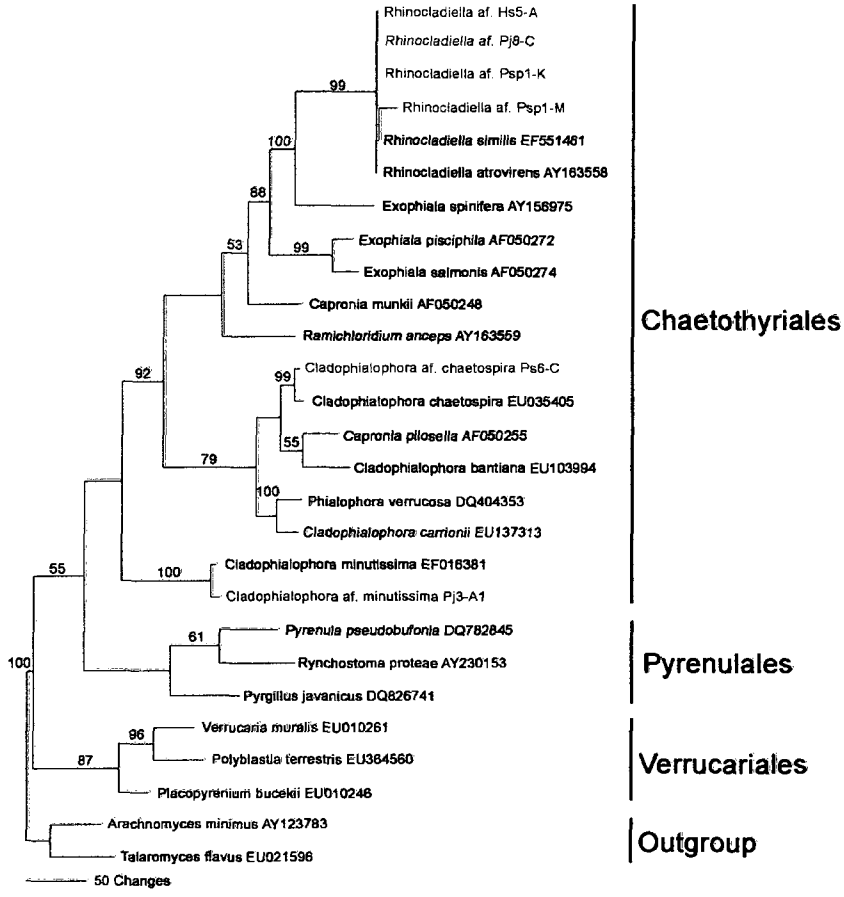
Rhinocladiella af. Hs5-A
Rhinocladiella af. Pj8-C
Rhinocladiella af. Psp1-K
Rhinocladiella af. Psp1-M
Rhinocladiella similis EF551461
Rhinocladiella atrovirens AY163558
Exophiala spinifera AY156975
Exophiala pisciphila AF050272
Exophiala salmonis AF050274
Capronia munkii AF050248
Ramichloridium anceps AY163559
Cladophialophora af. chaetospira Ps6-C
Cladophialophora chaetospira EU035405
Capronia pilosella AF050255
Cladophialophora bantiana EU103994
Phialophora verrucosa DQ404353
Cladophialophora carrionii EU137313
Cladophialophora minutissima EF016381
Cladophialophora af. minutissima Pj3-A1
Pyrenula pseudobufonia DQ782845
Rynchostoma proteae AY230153
Pyrgillus javanicus DQ826741
Verrucaria muralis EU010261
Polyblastia terrestris EU384560
Placopyrenium bucekii EU010246
Arachnomyces minimus AY123783
Talaromyces flavus EU021596
50 Changes
Chaetothyriales
Pyrenulales
Verrucariales
Outgroup

Figure 3-2. One of 2 equally parsimonious trees (1184 steps, CI=0.435, RI=0.603, HI=0.565) showing the placement of bryophilous fungi among the Dothideomycetes. Trees were inferred from maximum parsimony analysis of the 5.8S and ITS2 regions. The analysis included 453 characters, of which 196 were constant, 66 were uninformative, and 191 were parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

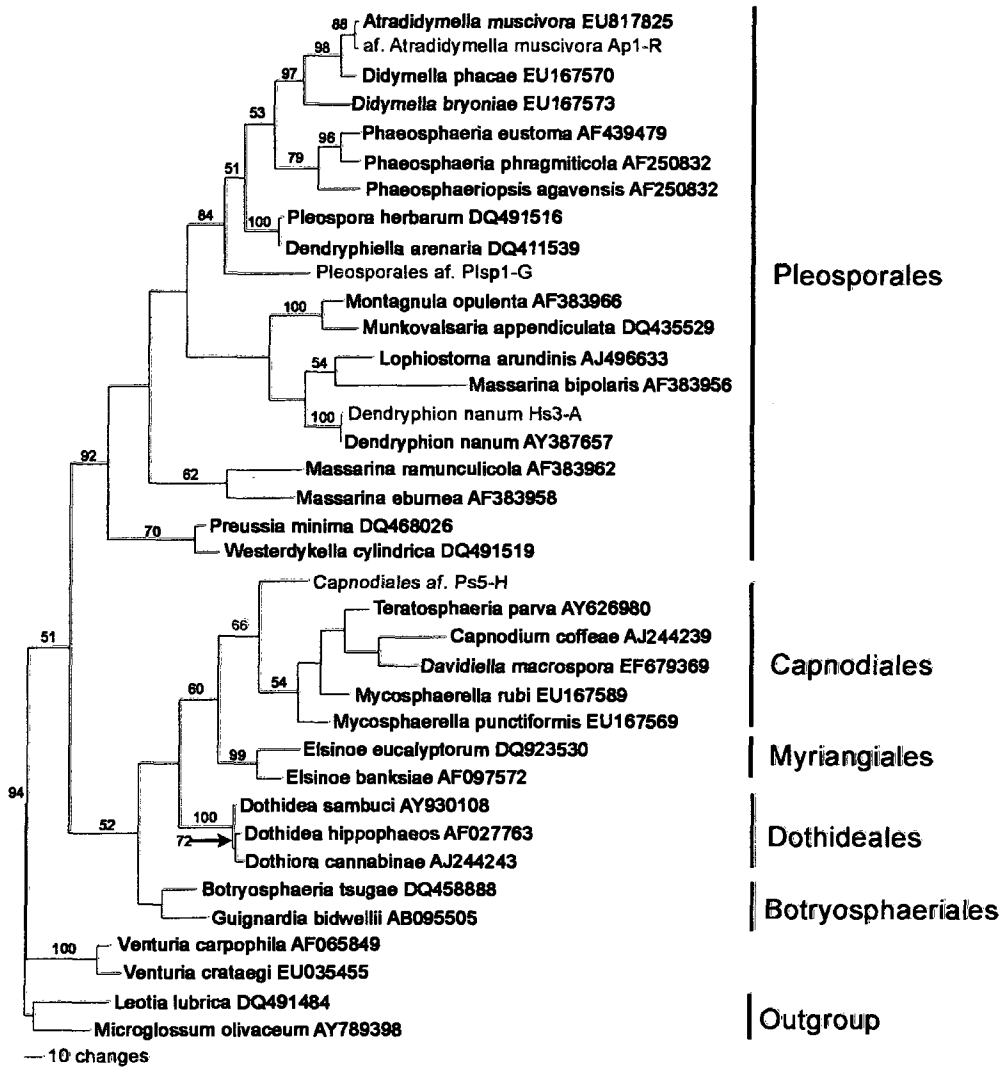
Atradidymella muscivora EU817825
af. Atradidymella muscivora Ap1-R
Didymella phacae EU167570
Didymella bryoniae EU167573
Phaeosphaeria eustoma AF439479
Phaeosphaeria phragmiticola AF250832
Phaeosphaeriopsis agavensis AF250832
Pleospora herbarum DQ491516
Dendryphiella arenaria DQ411539
Pleosporales af. Plsp1-G
Montagnula opulenta AF383966
Munkovalsaria appendiculata DQ435529
Lophiostoma arundinis AJ496633
Massarina bipolaris AF383956
Dendryphion nanum Hs3-A
Dendryphion nanum AY387657
Massarina ramunculicola AF383962
Massarina eburnea AF383958
Preussia minima DQ468026
Westerdykella cylindrica DQ491519
Capnodiales af. Ps5-H
Teratosphaeria parva AY626980
Capnodium coffeae AJ244239
Davidiella macrospora EF679369
Mycosphaerella rubi EU167589
Mycosphaerella punctiformis EU167569
Elsinoe eucalyptorum DQ923530
Elsinoe banksiae AF097572
Dothidea sambuci AY930108
Dothidea hippophaeos AF027763
Dothiora cannabinae AJ244243
Botryosphaeria tsugae DQ458888
Guignardia bidwellii AB095505
Venturia carpophila AF065849
Venturia crataegi EU035455
Leotia lubrica DQ491484
Microglossum olivaceum AY789398
10 changes
Pleosporales
Capnodiales
Myriangiales
Dothideales
Botryosphaeriales
Outgroup

Figure 3-3. One of 7 equally parsimonious trees (1132 steps, CI=0.529, RI=0.601, HI=0.471) showing the placement of bryophilous fungi among the Leotiomycetes. Trees were inferred from maximum parsimony analysis of ITS sequences. The analysis included 602 characters, of which 278 were constant, 74 were uninformative, and 250 were parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

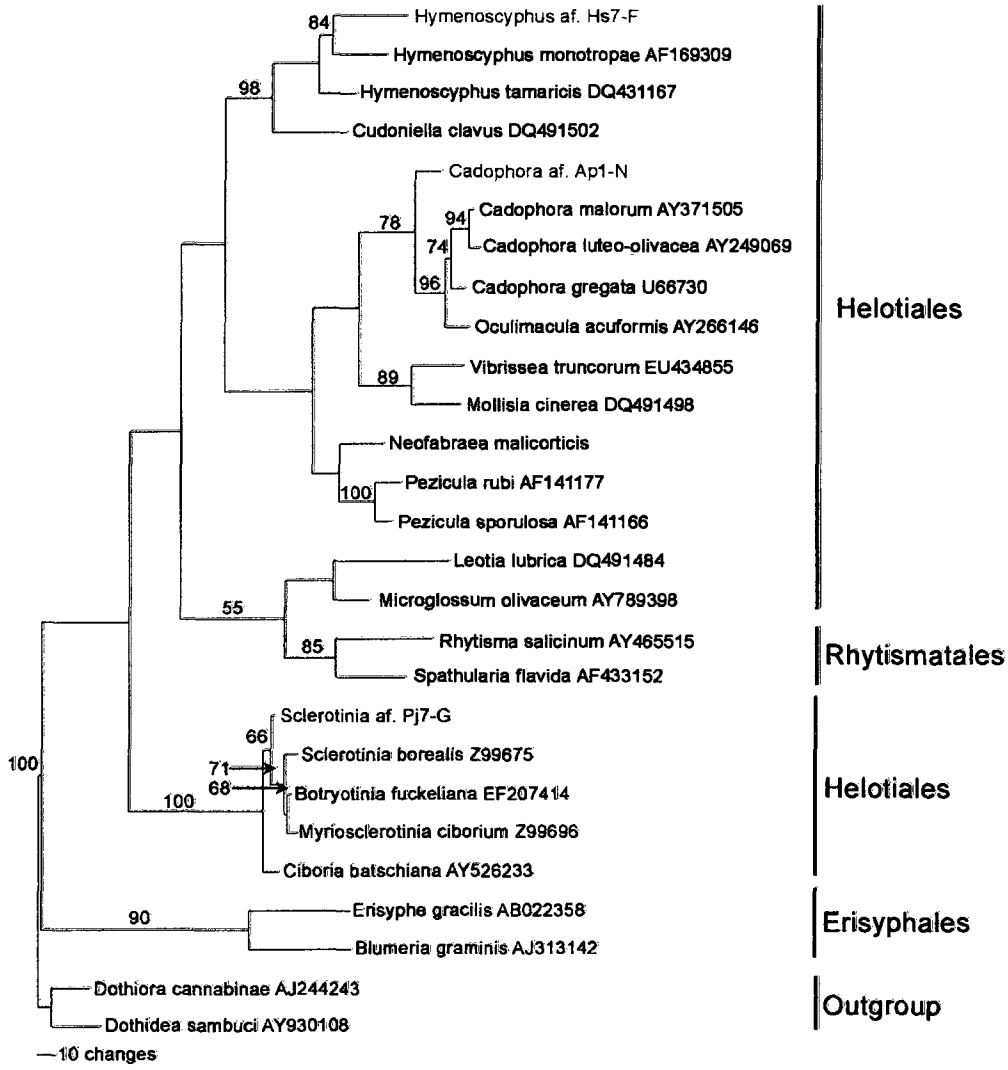
Hymenoscyphus af. Hs7-F
Hymenoscyphus monotropae AF169309
Hymenoscyphus tamaricis DQ431167
Cudoniella clavus DQ491502
Cadophora af. Ap1-N
Cadophora malorum AY371505
Cadophora luteo-olivacea AY249069
Cadophora gregata U66730
Oculimacula acuformis AY266146
Vibrissea truncorum EU434855
Mollisia cinerea DQ491498
Neofabraea malicorticis
Pezicula rubi AF141177
Pezicula sporulosa AF141166
Leotia lubrica DQ491484
Microglossum olivaceum AY789398
Rhytisma salicinum AY465515
Spathularia flavida AF433152
Sclerotinia af. Pj7-G
Sclerotinia borealis Z99675
Botryotinia fuckeliana EF207414
Myriosclerotinia ciborium Z99696
Ciboria batschiana AY526233
Erisyphe gracilis AB022358
Blumeria graminis AJ313142
Dothiora cannabinae AJ244243
Dothidea sambuci AY930108
10 changes
84
98
78
94
74
96
89
100
55
85
66
71
68
100
100
90
Helotiales
Rhytismatales
Helotiales
Erisyphales
Outgroup

Figure 3-4. Single most parsimonious tree (778 steps, CI=0.644, RI= 0.561, HI=0.356) showing the placement of bryophilous fungi among the Xylariomycetidae (Sordariomycetes). Trees were inferred from maximum parsimony analysis of ITS sequences. The analysis included 579 characters, of which 65 were constant, 126 were uninformative, and 188 were parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

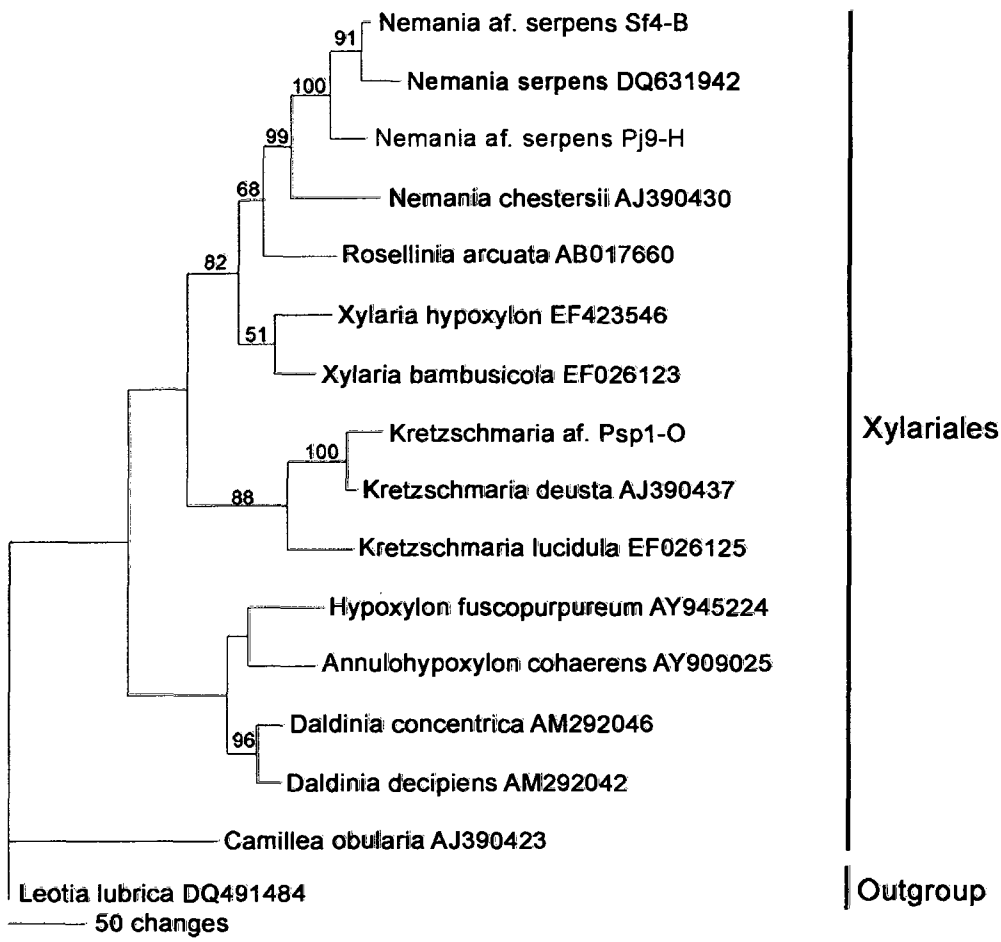
Nemania af. serpens Sf4-B
Nemania serpens DQ631942
Nemania af. serpens Pj9-H
Nemania chestersii AJ390430
Rosellinia arcuata AB017660
Xylaria hypoxylon EF423546
Xylaria bambusicola EF026123
Kretzschmaria af. Psp1-O
Kretzschmaria deusta AJ390437
Kretzschmaria lucidula EF026125
Hypoxylon fuscopurpureum AY945224
Annulohypoxylon cohaerens AY909025
Daldinia concentrica AM292046
Daldinia decipiens AM292042
Camillea obularia AJ390423
Leotia lubrica DQ491484
50 changes
91
100
99
68
82
51
100
88
96
Xylariales
Outgroup

Figure 3-5. One of 11 equally parsimonious trees (1195 steps, CI=0.589, RI=0.580, HI=0.411) showing the placement of bryophilous fungi among the Sordariomycetidae (Sordariomycetes). Trees were inferred from maximum parsimony analysis of ITS sequences. The analysis included 727 characters, of which 349 were constant, 131 were uninformative, and 247 were parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

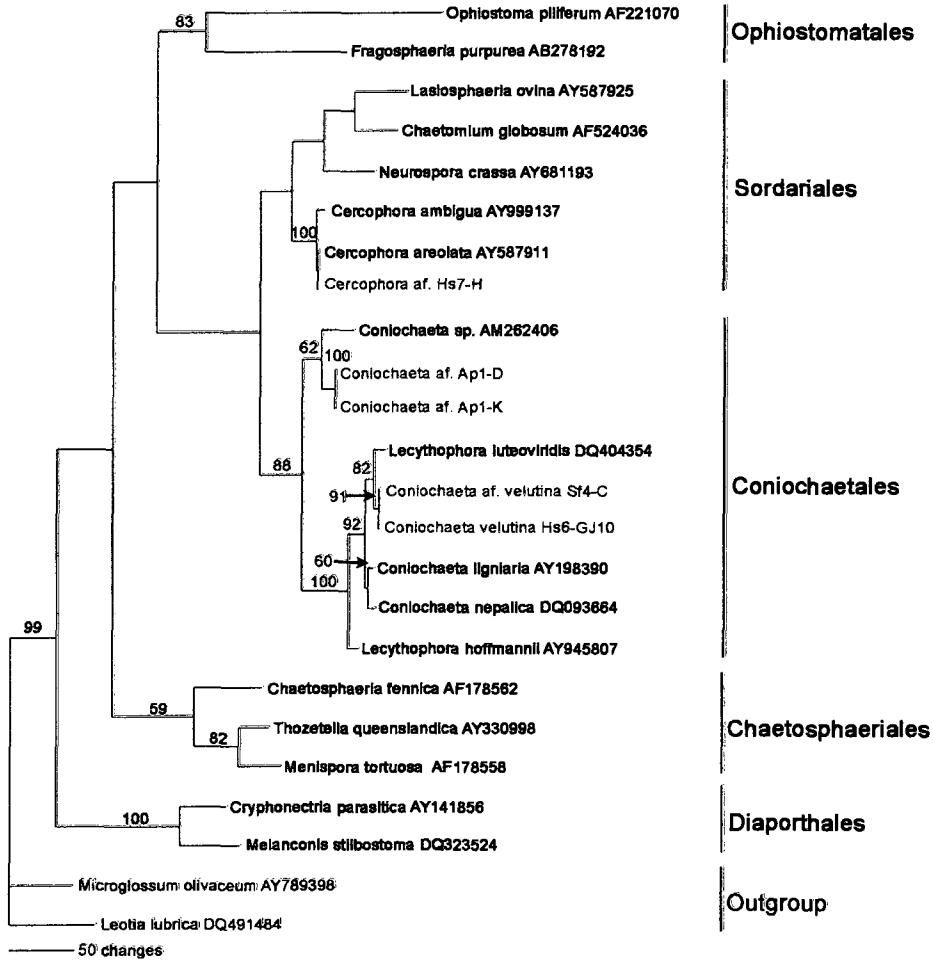
Ophiostoma piliferum AF221070
Fragosphaeria purpurea AB278192
Lasiosphaeria ovina AY587925
Chaetomium globosum AF524036
Neurospora crassa AY681193
Cercophora ambigua AY999137
Cercophora areolata AY587911
Cercophora af. Hs7-H
Coniochaeta sp. AM262406
Coniochaeta af. Ap1-D
Coniochaeta af. Ap1-K
Lecythophora luteoviridis DQ404354
Coniochaeta af. velutina Sf4-C
Coniochaeta velutina Hs6-GJ10
Coniochaeta ligniaria AY198390
Coniochaeta nepalica DQ093664
Lecythophora hoffmannii AY945807
Chaetosphaeria tennica AF178562
Thozetella queenslandica AY330998
Menispora tortuosa AF178558
Cryphonectria parasitica AY141856
Melanconis stilbostoma DQ323524
Microglossum olivaceum AY789396
Leotia lubrica DQ491484
50 changes
Ophiostomatales
Sordariales
Coniochaetales
Chaetosphaeriales
Diaporthales
Outgroup

Figure 3-6. Single most parsimonious tree (1247 steps, CI=0.579, RI=0.700, HI=0.421) showing the placement of bryophilous fungi among the Hypocreomycetidae (Sordariomycetes). Trees were inferred from maximum parsimony analysis of ITS sequences. The analysis included 727 characters, of which 343 were constant, 95 were uninformative, and 289 werer parsimony informative. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Sequences of bryophilous isolates from this study are shown in green type.

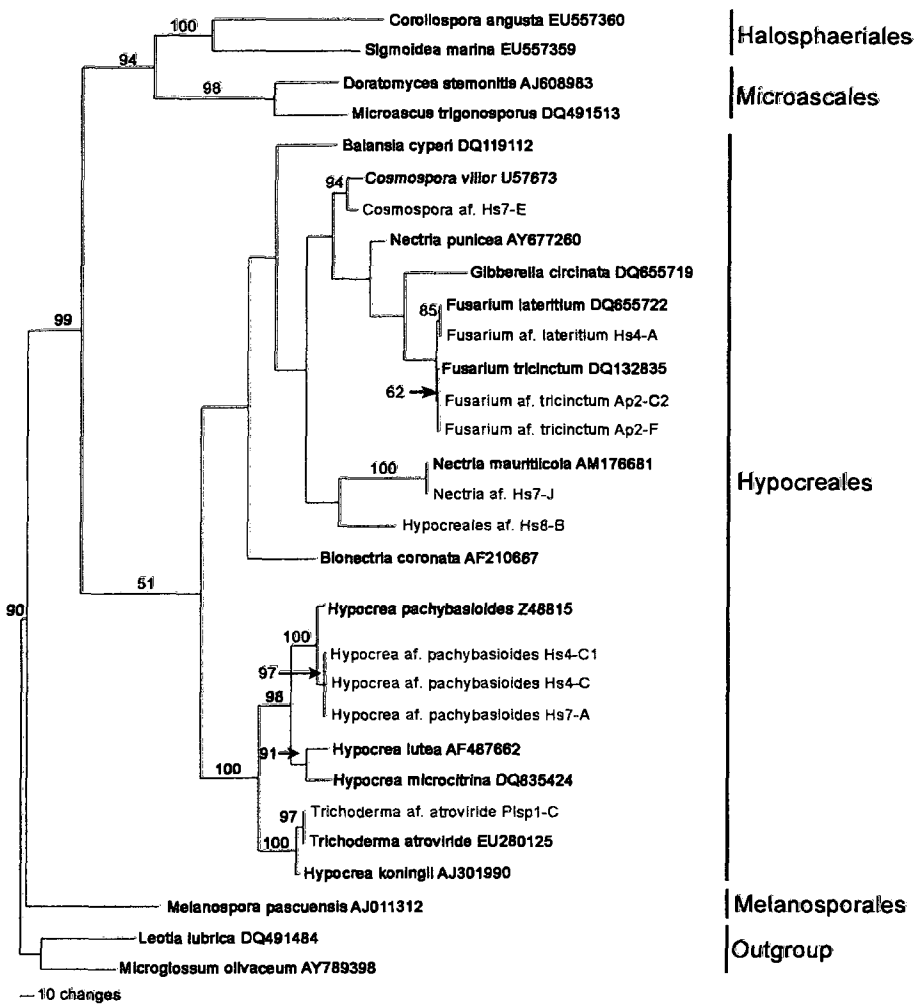
Corollospora angusta EU557360
Sigmoidea marina EU557359
Doratomyces stemonitis AJ608983
Microascus trigonosporus DQ491513
Balansia cyperi DQ119112
Cosmospora vilior U57673
Cosmospora af. Hs7-E
Nectria punicea AY677260
Gibberella circinata DQ655719
Fusarium lateritium DQ655722
Fusarium af. lateritium Hs4-A
Fusarium tricinctum DQ132835
Fusarium af. tricinctum Ap2-C2
Fusarium af. tricinctum Ap2-F
Nectria mauritiicola AM176681
Nectria af. Hs7-J
Hypocreales af. Hs8-B
Bionectria coronata AF210667
Hypocrea pachybasioides Z48815
Hypocrea af. pachybasioides Hs4-C1
Hypocrea af. pachybasioides Hs4-C
Hypocrea af. pachybasioides Hs7-A
Hypocrea lutea AF487662
Hypocrea microcitrina DQ835424
Trichoderma af. atroviride Plsp1-C
Trichoderma atroviride EU280125
Hypocrea koningii AJ301990
Melanospora pascuensis AJ011312
Leotia lubrica DQ491484
Microglossum olivaceum AY789398
10 changes
Halosphaeriales
Microascales
Hypocreales
Melanosporales
Outgroup

Table 3-1. Collection locales and host species collected.

Site #	Location	Description	Host Species Collected
1	**Moe's Bog** M SW6-60-6 W4 2 km west of Hoselaw, Alberta	Sphagnum fuscum bog with overstory of *Picea mariana* and *Larix laricina*	*Aulacomnium palustre* *Hylocomium splendens* *Pleurozium schreberi* *Polytrichum juniperinum* *Ptillium crista-castrensis* *Pylaisiella polyantha* *Sphagnum fuscum*
2	**Snagwood** Vicinity of Elk Island National Park SW16-54-19 W4 2 km east of eastern border of Elk Island National Park, Alberta	*Sphagnum* dominated wetland surrounded by aspen dominated upland	*Aulacomnium palustre* *Hylocomium splendens* *Polytrichum juniperinum* *Pylaisiella polyantha* *Sphagnum fuscum*
3	**Wolf Lake, Alberta** NW 35-65-7 W4 70 km northwest of Bonnyville, Alberta on the south shore of Wolf Lake	Wet lakeshore with overstory of *Picea glauca* interspersed with stands of *Betula papyrifera*	*Hylocomium splendens* *Polytrichum juniperinum*
4	**Redwater Provincial Recreation Area** NE 20-57-20 W4 10 km east of Redwater, Alberta	*Sphagnum* dominated wetland surrounded by *Pinus banksiana* dominated uplands	*Aulacomnium palustre* *Hylocomium splendens* *Polytrichum juniperinum* *Sphagnum squarrosum*
5	**Jackfish Lake Bog, Alberta** Along HWY 2, 7km east of Jackfish Lake	Mixed *Sphagnum* bog with overstory of *Picea mariana*	*Aulacomnium palustre* *Hylocomium splendens* *Sphagnum fuscum*
6	**Keheewin Lake, Alberta** SE 23 58 7 W4 12 km north of Elk Point, Alberta, 2 km west of the west shore of Keheewin Lake	Mesic *Populus balsamifera* and *Populus tremuloides* dominated uplands	*Hylocomium splendens* *Pleurozium schreberi* *Pylaisiella polyantha*
7	**Perryvale Bog, Alberta** 58°28' N, 113°16' W	*Sphagnum fuscum* dominated bog with overstory of *Picea mariana* and *Larix laricina*	*Polytrichum juniperinum* *Pylaisiella polyantha* *Sphagnum fuscum*
8	**Beauport Recreation Area, Quebec** 7km north of Quebec City on the west	Mesic forest with overstory of *Picea glauca, Fagus* sp., and *Acer saccharum.*	*Polytrichum alpinum* *Plagiomnium cuspidatum*

	shore of the Montmorency River.		
9	**Donahue's Pastures** SW21-59-10 W4 3km W-SW of Blue Ridge, Alberta	*Sphagnum fuscum* bog with overstory of *Picea mariana* that was selectively logged of large trees ten years previously	*Hylocomium splendens* *Polytrichum juniperinum* *Aulacomnium palustre* *Sphagnum fuscum*
10	**Thompson Creek, Alberta** NE 15-35-19 W5 7km east of Saskatchewan crossing, along the eastern banks of Thompson Creek	Mesic forest with overstory of *Pinus contorta*, *Picea glauca,* and *Abies balsamea*	*Hylocomium splendens*
11	**Moose Lake, Alberta** SW 16-61-6 W4 10 km soutwest of Bonnyville, Alberta, on the south shore of Moose Lake	Mesic lakeshore with overstory of *Populus balsamifera, Populus tremuloides* and *Betula papyrifera*	*Brachythecium velutinum* *Pleurozium schreberi* *Pylaisiella polyantha*

Table 3-2. Isolates sequenced in this study.

Order	Species	Isolate	Host
Capnodiales	Capnodiales aff.	Ps5-H	*Pleurozium schreberi*
Chaetothyriales	*Cladophialophora aff. chaetospira*	Ps6-C	*Pleurozium schreberi*
	Cladophialophora aff. *minutissima*	Pj3-A1	*Polytrichum juniperinum*
	Rhinocladiella aff.	Hs5-A	*Hylocomium splendens*
	Rhinocladiella aff.	Pj9-C	*Polytrichum juniperinum*
	Rhinocladiella aff.	Psp1-K	*Polytrichastrum alpinum*
	Rhinocladiella aff.	Psp1-M	*Polytrichastrum alpinum*
Coniochaetales	*Coniochaeta* aff.	Ap1-D	*Aulacomnium palustre*
	Coniochaeta aff.	Ap1-K	*Aulacomnium palustre*
	Coniochaeta aff. *velutina*	Sf4-C	*Sphagnum fuscum*
	Coniochaeta velutina	Hs6-GJ10	*Hylocomium splendens*
Helotiales	*Cadophora* aff.	Ap1-N	*Aulacomnium palustre*
	Hymenoscyphus aff.	Hs7-F	*Hylocomium splendens*
	Sclerotinia aff.	Pj7-G	*Polytrichum juniperinum*
Hypocreales	*Cosmospora* aff.	Hs7-E	*Hylocomium splendens*
	Fusarium aff. *lateritium*	Hs4-A	*Hylocomium splendens*
	Fusarium aff. *tricinctum*	Ap2-C2	*Aulacomnium palustre*
	Fusarium aff. *tricinctum*	Ap2-F	*Aulacomnium palustre*
	Hypocrea aff. *pachybasioides*	Hs4-C	*Hylocomium splendens*
	Hypocrea aff. *pachybasioides*	Hs4-C1	*Hylocomium splendens*
	Hypocrea aff. *pachybasioides*	Hs7-A	*Hylocomium splendens*
	Nectria aff.	Hs7-J	*Hylocomium splendens*
	Nectria aff.	Hs8-B	*Hylocomium splendens*
	Trichoderma aff. *atroviride*	Plsp1-C	*Plagiomnium cuspidatum*
Pleosporales	aff. *Atradidymella muscivora*	Ap1-R	*Aulacomnium palustre*
	Dendryphion nanum	Hs3-A	*Hylocomium splendens*
	Pleosporales aff.	Plsp1-G	*Plagiomnium cuspidatum*
Sordariales	*Cercophora* aff.	Hs7-H	*Hylocomium splendens*
Xylariales	*Kretzschmaria* aff	Psp1-O	*Polytrichastrum alpinum*
	Nemania aff. *serpens*	Sf4-B	*Sphagnum fuscum*
	Nemania aff. *serpens*	Pj9-H	*Polytrichum juniperinum*

Table 3-3. Sporulating fungal species observed and isolated as associates of forest bryophytes in Canada.

Order	Species	Host	Observed/ Isolated	Previous Reports
Capnodiales	*Cladosporium herbarum*	Ap, Hs, Pls, Pj, Pyp, Sf	O/I	Tosi et al. 2002 Thormann et al. 2001
Chaetothyriales	*Cladophialophora minutissima*	Ap, Pj, Sf	I	Davey & Currah 2007
	Phialophora sp.	Pj	I	Thormann and Rice 2007
	Rhinocladiella atrovirens	Pj, Sf	I	
	Ramichloridium anceps	Ap, Hs, Pj, Pls	I	
Coniochaetales	*Coniochaeta velutina*	Hs	I	
	Lecythophora sp.	Ap, Pls	I	
Dothideales	*Monodictys levis*	Pyp	I	
	Scleroconidioma sphagnicola	Hs	O/I	Tsuneda et al. 2000
Dothideomycetidae	*Bryochiton sp.*	Pj	O	Döbbeler 1978
Incertae sedis	*Epibryon sp.*	Pj, Pt	O	Döbbeler 1978
	Epibryon interlamellaris	Pj	O	Döbbeler 1978
Erysiphales	*Phyllactinia sp.*	Pls	O	
Eurotiales	*Aspergillus flavus*	Ap, Sf	I	
	Aspergillus fumigatus	Ap, Pyp, Sf	I	
	Paecilomyces farinosus	Hs, Pa, Pj, Pls, Pyp, Ss	I	Tosi et al. 2002
	Paecilomyces variottii	Pls	I	Möller and Dreyfuss 1996
	Penicillium citrinum	Pj	I	
	Penicillium frequentans	Ap, Hs, Pj, Sf, Ss	I	
	Penicillium nigricans	Pc	I	
	Penicillium thomii	Ap, Pj, Sf	I	Thormann et al. 2001 Rice and Thormann 2007
	Talaromyces flavus	Pj	I	
	Thysanophora penicilloides	Pj	I	
Hypocreales	*Acremonium cf. strictum*	Hs, Pj	I	Thormann et al. 2001
	Fusarium sporotrichioides	Hs	I	
	Lecanicillium lecanii	Hs, Pj	I	Thormann et al. 2001 Tosi et al. 2002
	Lecanicillium psalliotae	Pj	I	Thormann et al. 2001 Tosi et al. 2002
	Trichoderma harzianum	Pa	I	Thormann et al. 2001

	Trichoderma koningii	Sf, Ss	I	
	Trichoderma viride	Ap, Hs, Pa, Pj, Pyp, Pls, Ss	I	McRae and Seppelt 1999 Thormann et al. 2001
	Trichothecium roseum	Pyp	I	
	Tritirachium dependens	Ap, Pj	I	
Melanosporales	*Sphaerodes fimicola*	Bv	I	
Microascales	*Cephalotrichum microsporum*	Pj	I	
	Cephalotrichum nanum	Pj	I	
Myxotrichaceae	*Geomyces pannorum*	Pls	I	Kerry 1990 Möller and Dreyfuss 1996 Tosi et al. 2002
	Myxotrichum sp.	Bv	I	
	Oidiodendron anamorph of Myxotrichum cancellatum	Pj	I	Rice and Thormann 2007
	Oidioidendron griseum	Pj	I	Rice and Thormann 2007
	Oidioidendron maius	Pj	I	Thormann et al. 2001 Rice and Thormann 2007
	Pseudogymnoascus roseus	Pj	I/O	Rice and Thormann 2007
Ophiostomatales	*Leptographium piriforme*	Pj	I	
	Sporothrix cf. schenckii	Pj	I/O	Rice and Thormann 2007
Pleosporales	*Alternaria tenuissima*	Hs	I/O	
	Alternaria alternata	Ap, Hs, Pyp	I/O	Thormann and Rice 2007
	Atradidymella muscivora	Ap, Hs, Pj	I/O	
	Dendryphion nanum	Hs	I	
	Epicoccum nigrum	Ap, Hs	I	Racovitza 1959 Kerry 1990
	Pseudocladosporium hachijoensis	Pj	I	
	Tetracrium sp.	Pj	O	
	Ulocladium oudemansii	Ap, Pj	I	
Sordariales	*Chaetomium crispatum*	Hs	I	
	Chaetomium sp.	Pj	I	
	Sordaria fimicola	Bv	I	Thormann et al. 2001
Sordariomycetidae incertae sedis	*Arthrinium phaeospermum*	Hs, Pc, Sf, Ss	I	

Legend: Ap: Aulacomnium palustre, Bv: Brachythecium velutinum, Hs: Hylocomium splendens, Pa: Polytrichastrum alpinum, Pc: Plagiomnium cuspidatum, Pj: Polytrichum juniperinum, Ps: Pleurozium schreberi, Pyp: Pylaisiella polyantha, Sf: Sphagnum fuscum, Ss: Sphagnum squarrosum. I: fungus was isolated in culture. O: fungus was observed on the moss surface.

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Chapter 4

A new species of *Cladophialophora* (Hyphomycetes) from boreal and montane bryophytes.[2]

Introduction

Cladophialophora is a dematiaceous hyphomycete genus that produces branched chains of ellipsoidal to fusiform conidia through blastic, acropetal conidiogenesis (Borelli 1980; Ho et al. 1999). The genus was initially erected to accommodate a species exhibiting both *Cladosporium*- and *Phialophora*-like conidiogenesis (Borelli 1980). *Cladophialophora* includes species with affinities in the Herpotrichiellaceae (Chaetothyriomycetes *sensu* Eriksson et al. 2003), as indicated by molecular data (Abliz et al. 2004; Caligiorne et al. 2005; de Hoog et al. 2000; Haase et al. 1999; Untereiner et al. 1995) and the production of *Cladophialophora*-like anamorphs by some species of *Capronia*, the only known teleomorphic genus in this family (Muller et al. 1987; Untereiner 2000; Untereiner et al. 1999). The generic concept has since evolved to encompass dematiaceous hyphomycetes phylogenetically affiliated with the Herpotrichiellaceae and other taxa in the Chaetothyriales and exhibiting *Cladosporium*-like conidiogenesis. Species of *Cladophialophora* are commonly found as plant saprophytes and endophytes (Iwatsu 1984; Vicente et al. 2001; Zeppenfeldt et al. 1994), although strains of most of these have also been reported as mammalian pathogens, most

[2] A version of this chapter has been published. Davey, M.L. and R.S. Currah. 2007. Mycological Research 111: 106-116.

commonly as agents of phaeohyphomycosis or chromoblastomycosis in humans (Haase et al. 1999; Vicente et al. 2001; Zeppenfeldt et al. 1994).

Bryophilous fungi are a poorly characterized group, most of which are known from sexual fruiting bodies observed directly on moss gametophytes *in vivo*. Although fungi have been isolated from bryophytes in only a few studies, surveys of microfungi from *Sphagnum fuscum* (Schimp.) Klinggr. in a southern boreal bog (Thormann et al. 2001) and a variety of moss species in Antarctica (Tosi et al. 2002), as well as individual reports of various bryophilous fungi (Davey and Currah 2006) suggest they are a taxonomically diverse group with potential ecological roles as saprobes, pathogens, and parasites. During a survey of bryophilous fungi, a series of isolates morphologically consistent with *Cladophialophora* were obtained from *Aulacomnium palustre* (Hedw.) Schwaegr., *Polytrichum juniperinum* Hedw., and *Sphagnum fuscum* collected at four boreal or montane sites in central Alberta. *Cladophialophora minutissima* sp. nov. is erected below on the basis of morphological and molecular characters to accommodate these isolates.

Materials & Methods

Collection sites - Gametophytes of *Aulacomnium palustre*, *Polytrichum juniperinum* and *Sphagnum fuscum* were collected from four boreal or montane bogs in central Alberta (Table 4-1) along a 1000 km, roughly east-west transect.

Isolation - Individual gametophytes were cleaned of soil and debris by holding them upright under flowing tap water for 2-10 minutes. Gametophytes were then surface

sterilized by submerging for 1 minute in 1% NaOCl (diluted from 6% bleach (S.J. Whitaker Industries, Edmonton, Canada)), and rinsed three times by submerging for 2 minutes in sterile distilled water. Surface sterilized, and unsterilized, rinsed stems were placed in separate moist chambers (Pyrex petri dish [80 mm x 100 mm] containing 100 mL of perlite overlaid with two sheets of 90 mm diameter filter paper [Whatman, New Jersey, USA] and moistened with 10 mL of sterile distilled water). Individual shoots were cut into leaf and stem sections 5mm long, and surface sterilized as above. Fragments were plated on Mycosel (MYC: 36 g/L Mycosel Agar, Becton, Dickinson & Co., Sparks, MD, USA) and oatmeal agar (OA: 20g/L agar (Invitrogen, Carlsbad, CA, USA), 20 g/L ground oatmeal) amended with 0.01% oxytetracycline (Sigma, St. Louis, MO, USA). Moist chambers and isolation plates were incubated in the dark at 15°C and examined weekly for 6-8 weeks. Emerging hyphae were subcultured onto potato dextrose agar (PDA: 39 g/L potato dextrose agar, Difco Laboratories, Detroit, MI, USA), cornmeal agar (CMA: 17 g/L cornmeal agar, Acumedia Manufacturers, Baltimore, MD, USA), and OA. *Cladophialophora*-like isolates were obtained from both moist chambers and isolation plates after 2-4 weeks.

Characterization – To determine growth rates and colony morphology, single point inoculations of the ex-type strain made on OA, PDA, and CMA were incubated in the dark at 20°C, and observed every 10 days for one month. Colony measurements were calculated by averaging two perpendicular measurements of the diameter and are given as an average of 3 colonies. Microscopic morphology was examined on OA because this medium readily induces sporulation and promotes growth of aerial hyphae. Mounts of 5-

to 20-day-old slide cultures grown on cereal agar [CA: 100 g/L mixed cereal baby food (Heinz), 20 g/L agar] were made in lacto-fuchsin (0.1 g acid-fuchsin in 100 mL lactic acid) or polyvinyl alcohol with acid-fuchsin (0.05 g acid-fuchsin in 10 mL lactic acid and 1 mL glycerine mixed with 1.66 g of polyvinyl alcohol dissolved in 10 mL H_20) and light micrographs (LM) were taken using an Olympus BX50 microscope with a DP-12 digital camera. Measurements were made from 15-day-old slide cultures grown on CA. Twenty measurements of each structure were made and are expressed as: range (mean $\pm$ standard deviation). For scanning electron microscopy (SEM), 5 x 5 mm agar blocks were cut from 10 to 30 day-old cultures, vapour fixed with 2.5% glutaraldehyde in Millonig's buffer (pH 7.5) (Millonig 1961) for 16 hours, and vapour post-fixed with 1% OsO_4 in Millonig's buffer for 4 hours. Fixed material was dehydrated in an ethanol series, critical point dried (SeeVac Inc., Hialeah, FL, USA) using CO_2, coated with gold with an Edwards S150B sputter coater, and examined using a Hitachi S-2500 scanning electron microscope.

Phylogenetic analysis – Fungal isolates were grown on OA overlaid with a Cellophane™ membrane (UCB Films, Somerset, UK) for 30-50 days at ambient light and temperature. Genomic DNA was extracted from approximately 25 mg of mycelium as described by Cubero et al. (1999) with the following modifications. Material was disrupted by freezing samples with liquid nitrogen and then grinding them in a mortar and pestle with approximately 0.025g of acid washed sand and 1 mL of CTAB extraction buffer, and 2 µL of β-mercaptoethanol was added to this mixture prior to the 70°C incubation. The chloroform:isoamyl alcohol:extraction mixture was centrifuged for 20

minutes at 13 200 rpm and the aqueous phase purified using a QIAquick PCR Purification kit (Qiagen, Mississauga ON, Canada) instead of by alcohol precipitation. The internal transcribed spacer (ITS) and 18s small subunit ribosomal (SSU) regions were amplified as described by Gibas et al. (2004) using the forward and reverse primer sets ITS 4 (White et al. 1990) and BMB-CR (Lane et al. 1985), and NS1 and NS8 (White et al. 1990) respectively. PCR amplicons were purified using a QIAquick PCR Purification kit (Qiagen, Mississauga ON, Canada) and sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit v.3.0 (Applied Biosystems, Foster City, CA) using forward and reverse primers ITS1, ITS2, ITS3, ITS4, (White et al. 1990) and BMBC-R (Lane et al. 1985), and NS1, NS2, NS3, NS4, NS6, NS8 (White et al. 1990), NS13, and NS151 for the ITS and SSU regions, respectively. Sequencing reactions were run on an ABI 377 automated sequencer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Sequence identity was compared between strains using bl2seq (Tatusova and Madden 1999) for the ITS region. For sequence groups with 100% sequence identity, a single representative strain was included in data matrices for tree building. Data matrices of SSU sequences of members of the Chaetothyriomycetes and other major ascomycete classes, and ITS sequences of members of the Herpotrichiellaceae were created from sequences retrieved from GenBank. SSU sequences were aligned manually using the program SeAl v2.0a11 (Rambaut 1996), while ITS sequences were aligned using MAFFT v.5.8 (Katoh et al. 2005) and the subsequent alignment manually verified. These matrices were subjected to parsimony analysis using PAUP* v.4.0b10 (Swofford 2003) with simple step-wise addition of taxa,

tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Support for branching topologies was evaluated using 1000 resamplings of the data by bootstrapping analysis (Felsenstein 1985). All trees were scored for length in steps, consistency index (CI), retention index (RI), and homoplasy index (HI).

Enzyme activity – Representative isolates from each host species, and both the boreal and montane collection sites were tested for amylase, gelatinase, pectinase, cellulase, lipase, and insoluble and soluble polyphenolic oxidase enzyme activities using indicator media (Davidson et al. 1938; Hutchison 1990; Miyamoto et al. 2000). Indicator media were inoculated with 5 mm diameter plugs from 30-day-old cultures and incubated for 5 weeks. Lipase tests were incubated for 12 weeks.

In Vitro *Host Inoculation* – Mature, undehisced capsules of *Polytrichum juniperinum* were placed in 1.5 mL centrifuge tubes, surface sterilized in 1 mL of 1% NaOCl for one minute with agitation by vortexing, and then washed three times in 1 mL of sterile distilled water for one minute. Capsules were placed in 0.5 mL of sterile distilled water per capsule, ruptured with a sterile probe, and a spore suspension created by vortexing. The suspension was distributed evenly over the surface of 30 mL of White's media (Loveland 1956) solidified with 15g/L Phytagel (Sigma) in 175 mL glass tissue culture vessels (Sigma) with vented Magenta® B-cap closures (Sigma) (M. Plishka, Personal Communication 2005). Within 6-8 weeks of incubation at 15°C, and 75% relative humidity on a 12 h light, 12 h dark cycle, gametophytes reached the 8-12 leaf stage. Fungal spore suspensions were created by homogenizing 10 mm^2 samples of 30-day-old cultures of the ex-type strain in 1 mL of distilled water and vortexing.

Gametophytes were damaged by lightly crushing with forceps, and surface inoculated with either 200 µL of the spore suspension or 5 mm diameter plugs of 30-day-old cultures of the ex-type strain. Inoculated gametophytes, and uninoculated, control, gametophytes were incubated for 8 weeks and observed by LM every two weeks.

Results

Phylogenetic analysis – The aligned SSU data matrix consisting of sequences of *C. minutissima*, members of the Chaetothyriomycetes, and representatives of ascomycete classes sensu Eriksson et al. (2003) was composed of 43 taxa and included 1635 characters, of which 1146 were constant, 216 were parsimony uninformative, and 273 were parsimony informative.. Parsimony analysis generated 15 equally parsimonious trees, each with 997 steps (CI = 0.640, RI = 0.770, HI =0.360), whose topologies were consistent in their placement of strains of *C. minutissima*. Results of the bootstrap analysis are shown on one most parsimonious tree (Fig 4-1). Together with "Fungal sp. TRN527", an unidentified dematiaceous hyphomycete (GenBank Accession #: AY843287), isolates of *C. minutissima* form a monophyletic clade with strong bootstrap support (100%). Sequences of known Chaetothyriaceous anamorphs and species of *Capronia*, the single teleomorphic genus known in the Herpotrichiellaceae, form an unsupported clade that, in conjunction with the *C. minutissima* clade, compose the strongly supported (100%) Chaetothyriomycete clade. *Cladophialophora arxii* Tintelnot, *C. bantiana* (Sacc.) de Hoog, and *C. devriesii* (A.A. Padhye & Ajello) de Hoog, Kwon-Chung & McGinnis are present within a moderately well supported clade (75%) that also

includes *Fonsecaea monophora* (Moore and de Almeida) de Hoog, Vicente and Attili, *F. pedrosoi* (Brumpt) Negroni. The type species of *Cladophialophora, C. carrionii* (Trejos) de Hoog et al., forms a moderately well supported clade (83%) with *Capronia semiimmersa* (Cand. & Sulmont) Untereiner & F.A. Naveau, and *Phialophora verrucosa* Medlar. The phylogenetic position of *C. boppii* (Borelli) de Hoog, Kwon-Chung & McGinnis is unresolved within the Chaetothyriomycetes.

The aligned ITS matrix was composed of 65 taxa and included 747 characters, of which 276 were constant, 103 were parsimony uninformative, and 368 were parsimony informative. Parsimony analysis generated 8 equally parsimonious trees of 2967 steps (CI = 0.324, RI = 0.558, HI = 0.676) whose topologies were consistent in the position of the *C. minutissima* strains relative to other members of the Herpotrichiellaceae. Results of the bootstrap analysis are shown on one most parsimonious tree (Fig 4-2). Isolates of *C. minutissima* form a monophyletic clade with strong bootstrap support (93%) that groups with "Cladophialophora sp. TRN488", an unidentified dematiaceous hyphomycete (GenBank Accession #AY843173) with strong bootstrap support (100%). Within the *C. minutissima* clade, isolates are segregated into two groupings with bootstrap support of 96% and 92%, respectively. Sub-group A consists of all sequenced isolates from Site 1, while Sub-group B consists of all sequenced isolates from Sites 2, 3, and 4. Sequence identity comparison revealed 99% within-group sequence identity for both Sub-group A and B and 97% between-group sequence identity (Table 4-2). Current species of *Cladophialophora,* form a moderately well supported clade (70%) that is paraphyletic with the exclusion of *Phialophora verrucosa* and *Capronia semiimmersa.*

Within this clade, species form three moderate to strongly supported groups. The type species of the genus, *C. carrionii*, forms a well supported clade (94%) with *C. minourae* (Iwatsu) Haase & de Hoog (GenBank Accession #AY251087), *P. verrucosa* and *Ca. semiimmersa*. The species *C. bantiana, C. devriesii, C. arxii, C. minourae* (GenBank Accession #AB091213), and three unidentified species of *Cladophialophora* (GenBank Accession #AY857508, AY857507, AY857510) form a second well supported clade (93%) that groups with the *C. carrionii* clade with moderate support (67%). An unidentified species of *Cladophialophora* (GenBank Accession #AY781217) and *C. boppii* form a third, moderately supported (73%) clade that is basal to the other two clades. The phylogenetic position of the *C. minutissima* clade relative to the *Cladophialophora* clade does not receive bootstrap support >50%.

Enzyme activity – All isolates tested negatively for amylase, pectinase, cellulase, and soluble polyphenol oxidase enzyme activity. Tests for gelatinase and insoluble polyphenol oxidase activity were positive in all isolates.

In vitro *host inoculation* – After 4 weeks, inoculated gametophytes showed no difference in growth from uninoculated controls. Mycelium and conidia were evident on the surface of *Polytrichum juniperinum* leaves, stems, and protonemata, but hosts were asymptomatic, exhibiting no discoloration, morphological changes, or signs of penetration by the fungus.

Taxonomy

Cladophialophora minutissima M.L. Davey & R.S. Currah, sp. nov (Fig. 4-3 a-g)

MB 510074

Etym.: minutissima – refers to the very small conidia

Conidiophora micro-nematea vel mono-nematea, saepe solum tubera in hyphis vegetativis. Conidia unicellularia, rare monoseptata, cylindrica ad fusiformia cum hilis truncatis et crassis, pallide brunnea, tenuiter tunicata, levia, 1-2 x 8-22 µm, catenata, catenae interdum ramosae. Ramoconidia nulloseptata ad monoseptata, cylindrica ad fusiformia cum hilis multiplicibus et distalibus.

Typus: **Canada**: Alberta: 2 km ad orientem Elk Island Park, colonia exsiccata in agaro farinae avenae, isolata e foliis *Polytrichi juniperini* (musci) 03 May 2004, M.L. Davey (UAMH 10709 - holotypus)

Colonies on PDA, dark grey, sometimes fuscous, margin narrow, pale grey to olive, and reverse black. Colonies flat, smooth, with few aerial hyphae at first, becoming velvety, radially sulcate, convolute and cracked at center, raised 3-5 mm above agar surface, and attaining 25 mm diameter after 30 days at 20°C. On CMA, mycelium light grey, flocculose, margin grey to olive, reverse dark grey when young, becoming flat, grey, flocculose, margin dark grey to olive, reverse dark grey, attaining 20 mm diameter in 30 days. Aerial hyphae light grey or white, scattered in tufts. On OA, young colonies light grey, floccose, margin, if present, narrow and dark grey. Mature colonies flat, grey, floccose, margin, if present, grey to olive brown, 18 mm diameter in 30 days at 20°C (Fig 4-3).

Microscopic characters consistent on PDA, CMA, and OA. *Hyphae* mostly submerged in young cultures, pale to dark brown, smooth, septate, 1.5-2.5 µm wide.

Aerial hyphae abundant in older cultures; erect, branched, pale grey to white, septate, often with constrictions at septa, 1.5-2 μm wide. *Conidiophores* micronematous, poorly developed, often as protuberances on vegetative hyphae. *Conidia* unicellular, aseptate or rarely 1-septate, cylindrical to fusiform with truncate and thickened hila, pale brown, thin walled, smooth, 1-2 (1.7 ± 0.29) x 8-22 (13.9 ± 6.5) μm, catenate, chains occasionally branched. Ramoconidia 0-1 septate, cylindrical to fusiform with 2, rarely 3 distal hila, pale brown, 1.5-2 (1.9 ± 0.2) x 9-17 (12.4 ± 2.2) μm. Young conidia in chains 4-12 conidia long, resembling regularly septate hyphae constricted at septa; chains of mature conidia disarticulated, hila distinct. *Conidiogenesis* blastic, maturation acropetal, secession schizolytic (Fig 4-4 4-9).

Teleomorph: Not observed.

Additional specimens examined: **Canada,** Alberta, 2 km east of Elk Island Park, isolated from leaves and stems of *Sphagnum fuscum* (Bryophyta) 03 May 2004, M.L. Davey (UAMH 10710). Alberta, 3 km west-southwest of Blue Ridge, isolated from leaves and stems of *Sphagnum fuscum* (Bryophyta) 19 May 2004, M.L. Davey (UAMH 10711).

Discussion

Morphologically, isolates of *C. minutissima* are consistent with members of the genus *Cladophialophora* in producing slow growing, melanized hyphae that form micronematous conidiophores producing, catenate conidia in branched chains through blastic, acropetal conidiogenesis (Borelli 1980; Ho et al. 1999). The aseptate (rarely 1-septate) conidia with truncate scars resulting from schizolytic secession of the conidial

chains are also consistent with *Cladophialophora* species. However, isolates of *C. minutissima* are morphologically distinct from all previously described species of *Cladophialophora.* The new species can be distinguished from most others in the genus primarily by scar anatomy and conidium shape and size. The conidial scars of *C. minutissima* are characteristically thickened compared to those of other *Cladophialophora* species, although this character alone is not thought to be taxonomically significant (Ho et al. 1999). Conidia of *C. minutissima* are cylindrical to narrowly fusiform with a length:width ratio of 5 to 8:1, while conidia of *C. arxii, C. bantiana, C. carrionii, C. devriesii, C. hachijoensis, C. minourae* (Iwatsu) Haase & de Hoog, and *C. modesta* McGinnis, de Hoog & Haase are broadly fusiform to limoniform with length:width ratios ranging from 1.5:1 to 4:1 (Borelli 1980; Braun and Feiler 1995; Ho et al. 1999; Iwatsu 1984; McGinnis et al. 1999; Tintelnot et al. 1995). *C. minutissima* is further distinguished from *C. minourae* and *C. carrionii* by its ability to liquefy gelatin (Honbo et al. 1984; Iwatsu 1984). A dichotomous key to the species of *Cladophialophora,* including *C. minutissima,* is provided following this discussion.

SSU-based phylogenetic analysis (Fig. 4-1) places isolates of *C. minutissima* as a strongly supported clade within the Herpotrichiellaceae in the Chaetothyriomycetes (*sensu* Eriksson et al. 2003), which is consistent with the phylogenetic affinities of other *Cladophialophora* species (Abliz et al. 2004; Caligiorne et al. 2005; de Hoog et al. 2000, 2004; Haase et al. 1999). Although a teleomorph has not been observed, the inclusion of *C. minutissima* in a strongly supported clade with perithecial species of *Capronia*, the single teleomorphic genus known in the Herpotrichiellaceae, of which several species

have been reported to have *Cladophialophora*-like anamorphs (Muller et al. 1987; Untereiner et al. 1999, 2000), suggests that any teleomorph of these isolates would be similar to *Capronia*. The grouping of *C. minutissima* with "Fungal sp. TRN527", a dematiaceous hyphomycete from quartzite rock in the Central Mountain System of Spain (Ruibal 2004), with high bootstrap support (100%) suggests possible conspecificity; however, isolates of "Fungal sp. TRN527" and related strains from the same study are currently unavailable for further morphological and molecular comparison.

Maximum parsimony analysis of the ITS region supports morphological data that suggests *C. minutissima* is distinct from other species of *Cladophialophora* and also indicates genetic divergence among isolates based on geographic location. High bootstrap support for Subgroups A and B, in conjunction with direct sequence identity comparison indicating 99-100% within-group similarity and 97% between-group similarity, suggests significant divergence between the groups. Because intraspecific hypervariability of the ITS region is common among Herpotrichiellaceous species (Caligiorne et al. 1999, 2005), Subgroups A and B are still considered to be conspecific, and collectively representing the species *C. minutissima*.

The assignment of *C. minutissima* to *Cladophialophora* compounds the level of polyphyly in the genus. However, the binomial is proposed on the grounds of minimizing superfluous names. Given the prevalence of polymorphic conidiogenesis (Abliz et al. 2004; Borelli 1980), synanamorphy (de Hoog 1977, 1995a; Untereiner et al. 1995), relative lack of phenetic characters, high phenotypic plasticity, and high intraspecific genetic variability among anamorphic species in the Herpotrichiellaceae

(Caligiorne et al. 1999, 2005; de Hoog et al. 2000; Masclaux et al. 1996), all of which hinder identification and classification, erecting a new form genus that is almost morphologically indistinguishable from *Cladophialophora* species would only compound identification difficulties in a group already rife with problems. The systematics of the Herpotrichiellaceae should be re-evaluated. Herpotrichiellaceous teleomorphs are relatively easy to discern (Untereiner et al. 1995), and this feature, in conjunction with the development of phylogenetic analyses that accommodate high sequence variability, such as DNA-walk divergence (Caligiorne et al. 2005), could be used in a molecular-morphotaxonomic approach to systematics similar to that described by Untereiner et al. (1995). The taxonomy of isolates herein designated *Cladophialophora minutissima* would likely be re-evaluated in any such revision.

With its pale brown, slightly melanized hyphae and blastic, acropetal conidiogenesis producing cylindrical to fusiform conidia with truncate ends, *C. minutissima* superficially resembles *Heteroconium*, *Devriesia, Xylohypha*, *Cladosporium* and *Pseudocladosporium* and could be easily confused with members of these genera. However, *Heteroconium* species differ from *C. minutissima* in having macronematous conidiophores, and septate conidia in unbranched chains. Although consistent in conidium size, septation, and scar morphology, *C. minutissima* lacks the chlamydospore synanamorph, macronematous, sometimes branched conidiophores, and predilection for soil substrates common to species in *Devriesia* (Seifert et al. 2004). Furthermore, *Devriesia* species are affiliated with the Mycosphaerellaceae (Dothideomycetes) (Seifert et al. 2004). *Xylohypha* species are distinguished from *C. minutissima* by their thickened

conidial walls, dark melanization and lack of truncate, terminal conidial scars (Hughes and Sugiyama 1972). *Cladophialophora minutissima* and species of *Cladosporium* are consistent in conidiogenesis and terminal scar morphology, but inconsistent in conidiophore morphology, the latter producing macronematous conidiophores (de Hoog et al. 1995b). Furthermore, *Cladosporium* species are within the Mycosphaerellaceae (Dothideomycetes) (Braun et al. 2003; Masclaux et al. 1995). The long fusiform to cylindrical conidia and plant substrate of *C. minutissima* is consistent with species of *Pseudocladosporium* (Venturiaceae, Dothideomycetes), most of which were previously classified within *Cladophialophora* (Braun et al. 2003); however, *C. minutissima* is distinguishable from these species by its narrow (<2 μm), almost exclusively aseptate conidia, and phylogenetic affinities to the Chaetothyriomycetes.

The lack of discernible symptoms and evidence of host cell penetration by *C. minutissima* after inoculation on axenically grown *P. juniperinum* suggests that it is an opportunistic or saprobic fungus on its host mosses. Bryophytes have 'leaky cells' that release nutrient-rich aqueous leachates during wetting and drying cycles (Carleton and Read 1991), which would allow *C. minutissima* to use its hosts simply as a physical platform and opportunistic nutrient source. However, enzymatic tests indicating *C. minutissima* has polyphenol oxidase activity allowing it to degrade insoluble polyphenolic compounds like those found in moss cell walls (Popper and Fry 2003; Verhoeven and Liefveld 1997), in conjunction with the isolation of the fungus from surface sterilized mosses, suggests the fungus may be an endophyte, or at least a latent saprobe that actively degrades cell walls after host plant death. This ecological role

would be consistent with other members of *Cladophialophora* which exhibit the enzymatic capacity to degrade aromatic-ring based compounds (Prenafeta-Boldu et al. 2002, 2004), and are known as saprophytes and endophytes of dead and decaying plant material (Braun and Feiler 1995; Iwatsu 1984; Vicente et al. 2001; Zeppenfeldt et al. 1994). However, decomposition studies on dead host material and further examination for evidence of endophytic hyphae within host plants have yet to be undertaken to verify this potential ecological role of *Cladophialophora minutissima*. Furthermore, Herpotrichiellaceous fungi able to degrade aromatic compounds are often closely related to or conspecific with those strains of fungi pathogenic to mammals (Prenafeta-Boldu et al. 2006), suggesting that *C. minutissima* should also be evaluated for potential pathogenesis in animal systems.

Key to Species of *Cladophialophora*

1. Conidia sub-globose, ellipsoidal, limoniform, or short-fusiform (length:width ratio < 4:1).....2
1. Conidia long-fusiform or sub-cylindrical (length:width ratio > 4:1)..... .....*C. minutissima*

2. Conidia in short simple or branched chains (< 10 conidia per chain).....3
2. Conidia in long simple or branched chains (> 10 conidia per chain).....5

3. Branches of conidial chains sessile ..... *C. modesta*
3. Branches of conidial chains borne on short denticles.....4

4. Conidia 7-15 µm x 3-4 µm, able to grow at 40°C, and forming chlamydospores at low pH.....*C. arxii*
4. Conidia 5-6.5 µm x 2.5-3 µm, unable to grow at 40°C, and lacking chlamydospores.....*C. devriesii*

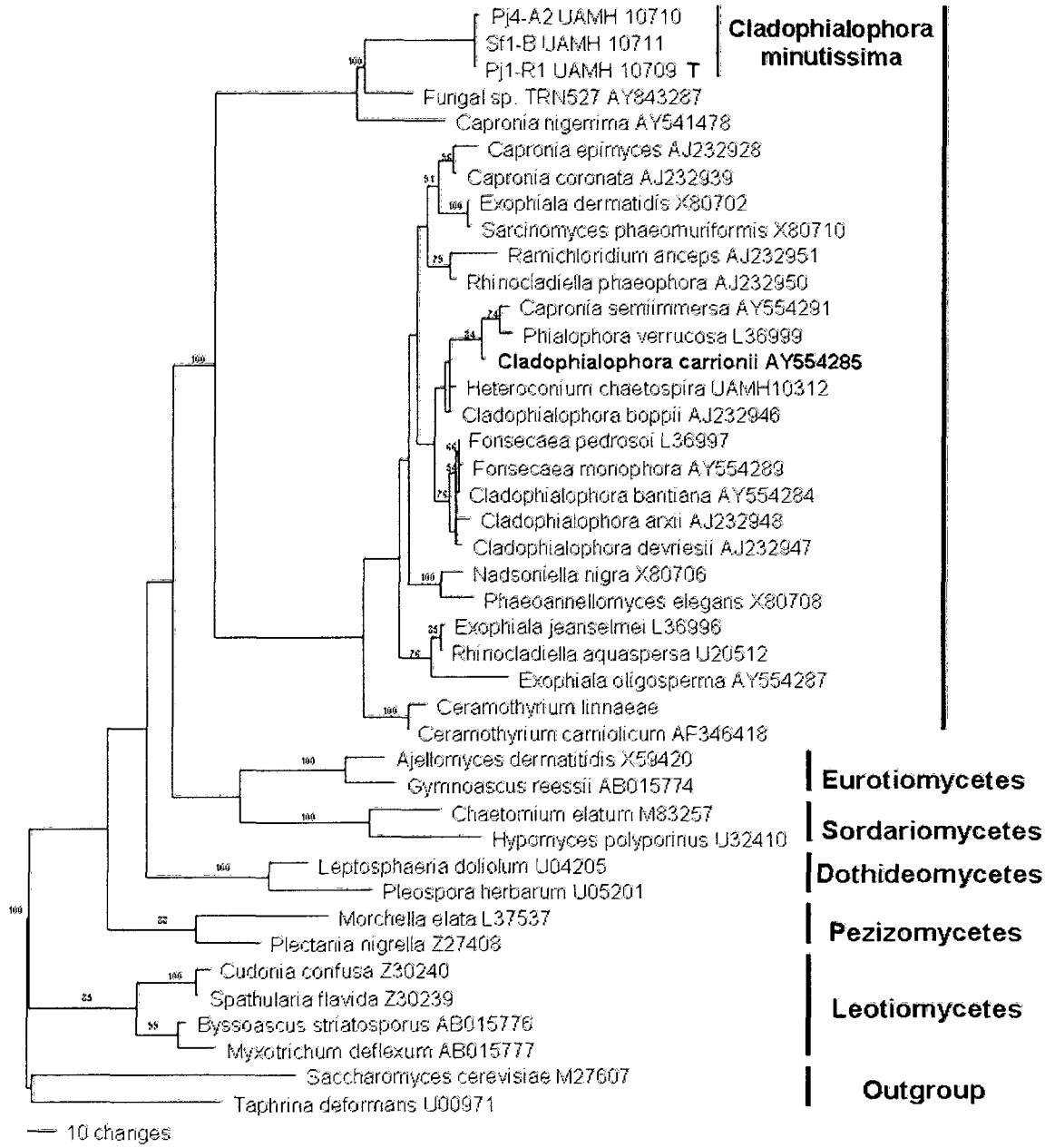
5. Conidia sub-globose and in simple chains.....*C. boppii*
5. Conidia ellipsoidal, limoniform, or short fusiform and in simple or branched chains.....6

6. *Phialophora*-like synanamorph present .....*C. carrionii*
6. *Phialophora*-like synanamorph absent.....7

7. Conidial chains profusely branched (> 4 times per conidiophore).....*C. minourae*
7. Conidial chains sparsely branched (< 4 times per conidiophore).....8

8. Conidia 2.5-3.5 µm x 7.5-8 µm, globose chlamydospores sometimes present .....*C. bantiana*
8. Conidia 2.5-3 µm x 5.5-7 µm, chlamydospores absent .....*C. carrionii*

Figure 4-1. One of 15 equally parsimonious trees (997 steps, CI=0.632, RI=0.768, HI=0.368) inferred from a maximum parsimony analysis of small subunit rDNA sequences showing the placement of isolates of *Cladophialophora minutissima* among the classes of ascomycetes and members of the Herpotrichiellaceae. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. *Taphrina deformans* (Berk.) Tul. and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen are outgroup taxa. Accession numbers for sequences retrieved from GenBank are given following species name. The type species of *Cladophialophora* is in bold, and the type strain of *C. minutissima* is denoted by '**T**' following the name and accession number.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Sf1-B UAMH 10711
Pj1-R1 UAMH 10709 T
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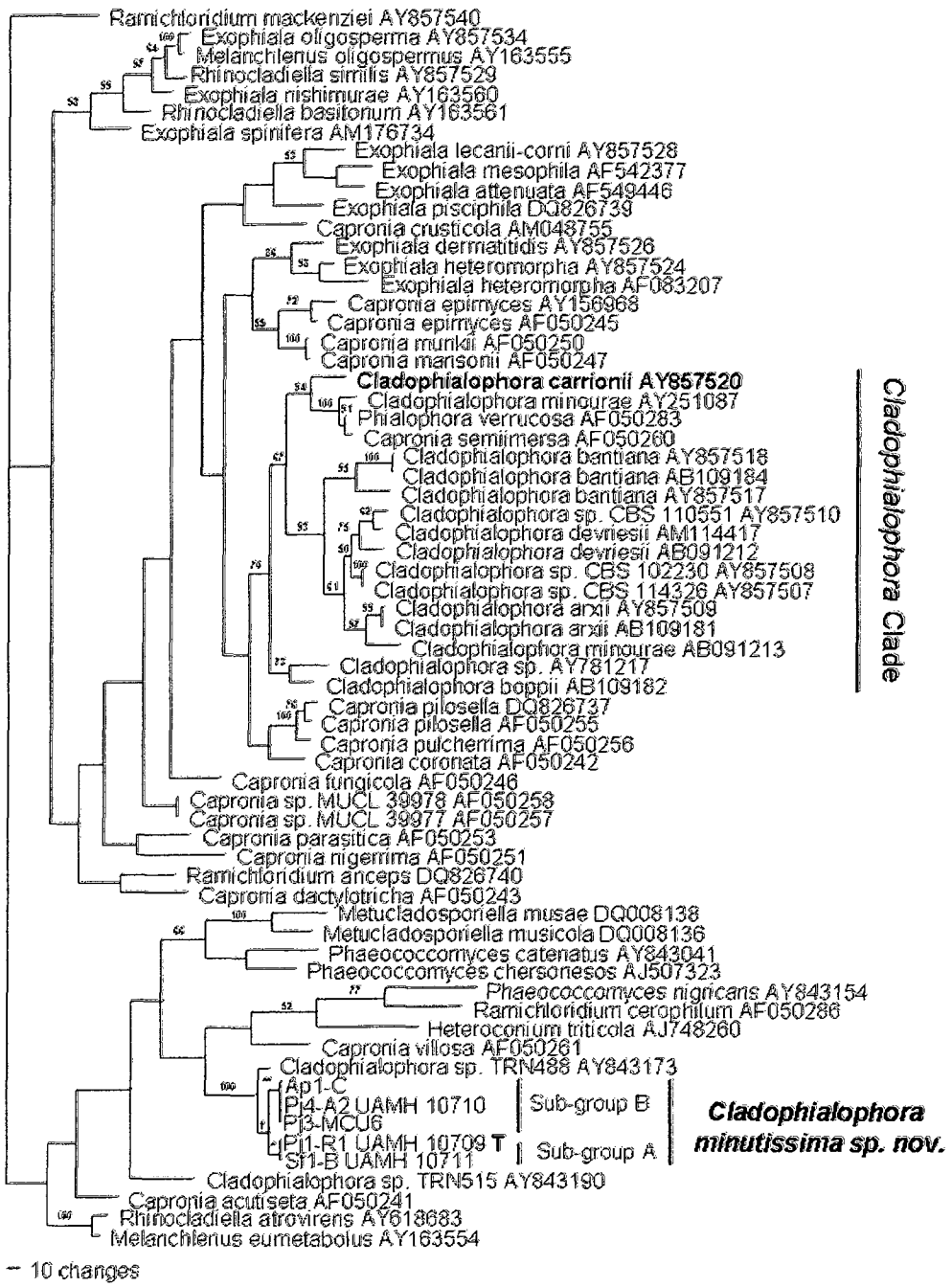
Fungal sp. TRN527 AY843287
Capronia nigerrima AY541478
Capronia epimyces AJ232928
Capronia coronata AJ232939
Exophiala dermatidis X80702
Sarcinomyces phaeomuriformis X80710
Ramichloridium anceps AJ232951
Rhinocladiella phaeophora AJ232950
Capronia semiimmersa AY554291
Phialophora verrucosa L36999
Cladophialophora carrionii AY554285
Heteroconium chaetospira UAMH10312
Cladophialophora boppii AJ232946
Fonsecaea pedrosoi L36997
Fonsecaea monophora AY554289
Cladophialophora bantiana AY554284
Cladophialophora arxii AJ232948
Cladophialophora devriesii AJ232947
Nadsoniella nigra X80706
Phaeoannellomyces elegans X80708
Exophiala jeanselmei L36996
Rhinocladiella aquaspersa U20512
Exophiala oligosperma AY554287
Ceramothyrium linnaeae
Ceramothyrium carniolicum AF346418
Ajellomyces dermatitidis X59420
Gymnoascus reessii AB015774
Chaetomium elatum M83257
Hypomyces polyporinus U32410
Leptosphaeria doliolum U04205
Pleospora herbarum U05201
Morchella elata L37537
Plectania nigrella Z27408
Cudonia confusa Z30240
Spathularia flavida Z30239
Byssoascus striatosporus AB015776
Myxotrichum deflexum AB015777
Saccharomyces cerevisiae M27607
Taphrina deformans U00971
10 changes
Eurotiomycetes
Sordariomycetes
Dothideomycetes
Pezizomycetes
Leotiomycetes
Outgroup

Fig 4-2. One of 8 equally parsimonious, unrooted trees (2967 steps, CI=0.324, RI=0.558, HI=0.676) inferred from a maximum parsimony analysis of internal transcribed spacer sequences showing the placement of isolates of *Cladophialophora minutissima* among members of the Herpotrichiellaceae. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. Accession numbers for sequences retrieved from GenBank are given following species name. '†' denotes 93% bootstrap support for the *C. minutissima* clade. '*'and '**' denote 96% and 92% bootstrap support for Sub-groups A and B of the *C. minutissima* clade, respectively. The type species of *Cladophialophora* is in bold, and the type strain of *C. minutissima* is denoted by '**T**' following the name and accession number.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Rhinocladiella basitonum AY163561
Exophiala spinifera AM176734
Exophiala lecanii-corni AY857528
Exophiala mesophila AF542377
Exophiala attenuata AF549446
Exophiala pisciphila DQ826739
Capronia crusticola AM048755
Exophiala dermatitidis AY857526
Exophiala heteromorpha AY857524
Exophiala heteromorpha AF083207
Capronia epimyces AY156968
Capronia epimyces AF050245
Capronia munkii AF050250
Capronia mansonii AF050247
Cladophialophora carrionii AY857520
Cladophialophora minourae AY251087
Phialophora verrucosa AF050283
Capronia semiimmersa AF050260
Cladophialophora bantiana AY857518
Cladophialophora bantiana AB109184
Cladophialophora bantiana AY857517
Cladophialophora sp. CBS 110551 AY857510
Cladophialophora devriesii AM114417
Cladophialophora devriesii AB091212
Cladophialophora sp. CBS 102230 AY857508
Cladophialophora sp. CBS 114326 AY857507
Cladophialophora arxii AY857509
Cladophialophora arxii AB109181
Cladophialophora minourae AB091213
Cladophialophora sp. AY781217
Cladophialophora boppii AB109182
Capronia pilosella DQ826737
Capronia pilosella AF050255
Capronia pulcherrima AF050256
Capronia coronata AF050242
Capronia fungicola AF050246
Capronia sp. MUCL 39978 AF050258
Capronia sp. MUCL 39977 AF050257
Capronia parasitica AF050253
Capronia nigerrima AF050251
Ramichloridium anceps DQ826740
Capronia dactylotricha AF050243
Metucladosporiella musae DQ008138
Metucladosporiella musicola DQ008136
Phaeococcomyces catenatus AY843041
Phaeococcomyces chersonesos AJ507323
Phaeococcomyces nigricans AY843154
Ramichloridium cerophilum AF050286
Heteroconium triticola AJ748260
Capronia villosa AF050261
Cladophialophora sp. TRN488 AY843173
Ap1-C
Pl4-A2 UAMH 10710
Pl3-MCU6
Pl1-R1 UAMH 10709 T
Sf1-B UAMH 10711
Sub-group B
Sub-group A
Cladophialophora minutissima sp. nov.
Cladophialophora Clade
Cladophialophora sp. TRN515 AY843190
Capronia acutiseta AF050241
Rhinocladiella atrovirens AY618683
Melanchlenus eumetabolus AY163554
10 changes

Figure 4-3 to 4-9. Colonial and microscopic morphology of ex-type strain (UAMH 10709) of *Cladophialophora minutissima* sp. nov.

Figure 4-3. Composite picture showing colonies on different media after 30 days incubation at 20°C. a. cornmeal agar b. oatmeal agar c. potato dextrose agar. Scale bar = 10 mm.

Figure 4-4. Light micrograph showing micronematous conidiophores integrated into vegetative hyphae and blastic, acropetal production of conidia. Scale bar = 15 μm.

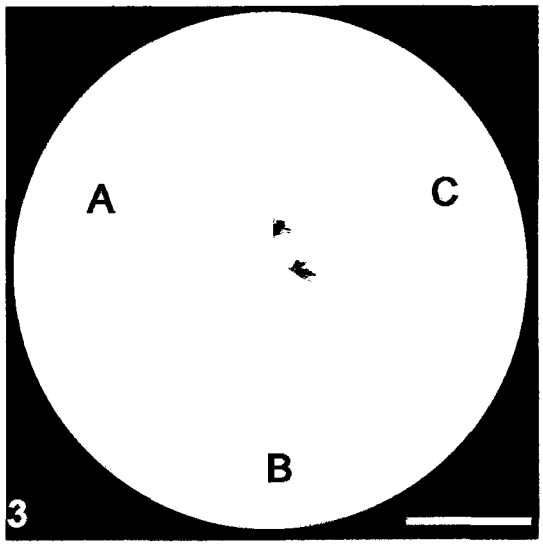
Figure 4-5. Cylindrical to fusiform, immature, catenate conidia (white arrowhead) and mature chain of disarticulated conidia (black arrowhead. Scale bar = 13 μm.

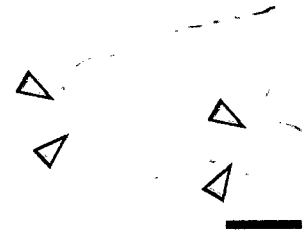
Figure 4-6. Mature, branched chain of conidia undergoing schizolytic secession. Arrowheads pointing to ramoconidia. Scale bar = 16 μm.

Figure 4-7. Disarticulated, seceded cylindrical to fusiform conidia of *C. minutissima.* Scale bar = 13 μm.

Figure 4-8. Immature, mature, and disarticulated conidia of *C. minutissima* with smooth walls and truncate, thickened conidial scars. Scale bar = 6 μm.

Figure 4-9. Branched chains of conidia of *C. minutissima* seceding schizolytically. Scale bar = 10 μm.


A
C
B
3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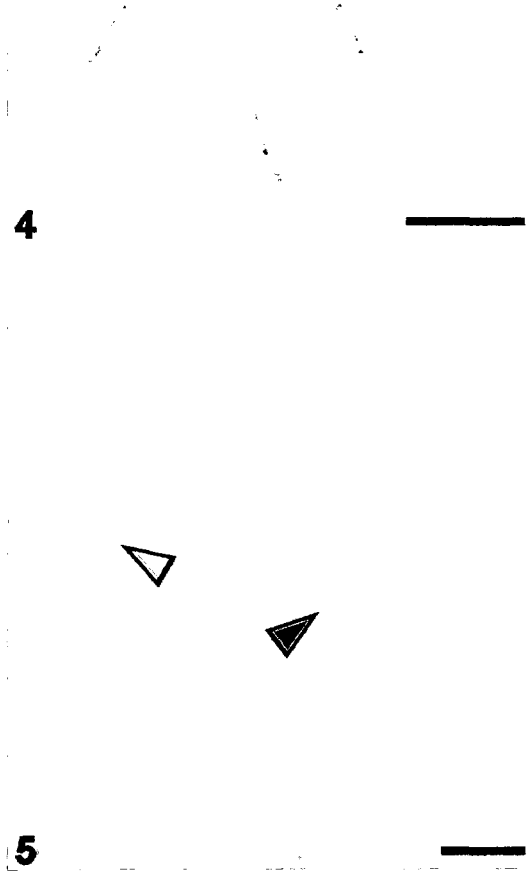
4
5

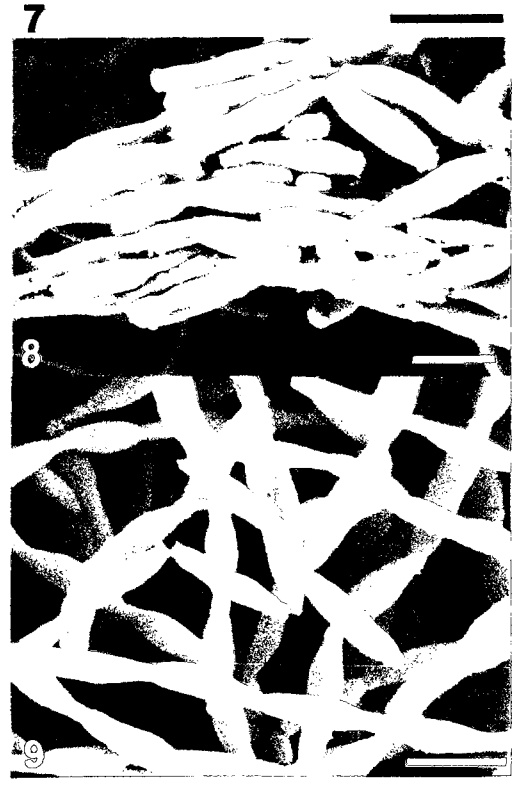
7
8
9

Table 4-1. Boreal and montane bog collection sites in central Alberta, Canada, yielding isolates of *Cladophialophora minutissima*.

Site #	Location	Description	Isolate	Host Moss Species
1	Vicinity of Elk Island National Park SW16-54-19 W4 2km east of eastern border of Elk Island National Park	*Sphagnum* dominated wetland surrounded by aspen dominated upland	Pj1-A1	*Polytrichum juniperinum*
			Pj1-B	*P. juniperinum*
			Pj1-B from MC	*P. juniperinum*
			Pj1-MCU1	*P. juniperinum*
			Pj1-MCU2	*P. juniperinum*
			Pj1-L1	*P. juniperinum*
			Pj1-L2	*P. juniperinum*
			Pj1-R1	*P. juniperinum*
			Sf1-B	*Sphagnum fuscum*
			Pj2-A1	*P. juniperinum*
			Pj2-B1	*P. juniperinum*
			Pj2-R2	*P. juniperinum*
2	Moe's Bog SW6-60-6 W4 2 km west of Hoselaw, Alberta	*Sphagnum fuscum* bog with overstory of *Picea mariana* and *Larix laricina*	Pj3-R1	*P. juniperinum*
			Pj3-R2	*P. juniperinum*
			Pj3-MCU6	*P. juniperinum*
3	Donahue's Pastures SW21-59-10 W4 3km W-SW of Blue Ridge, Alberta	*Sphagnum fuscum* bog with overstory of *Picea mariana* that was selectively logged of large trees 10 years previously	Pj4-A2	*P. juniperinum*
				P. juniperinum
				P. juniperinum
4	Jackfish Lake Bog Along HWY 2, 7km east of Jackfish Lake	Mixed *Sphagnum* bog with overstory of *Picea mariana*	Ap1-C	*Aulacomnium palustre*

Table 4-2. Pairwise ITS sequence identity for isolates of *Cladophialophora minutissima* isolated from four boreal and montane bog sites. Overall identity expressed as a percentage. Sequence differences expressed as gaps(G)/substitutions(S). Pale gray shaded areas represent Subgroup 1 and medium gray shaded areas represent Subgroup 2.

	Pj1-A1	**Pj1-B**	**Pj1-MCU2**	**Pj1-R1**	**Sf1-B**	**Pj3-MCU6**	**Pj4-A2**	**Ap1-C**
Pj1-A1	**100%** 0G/0S							
Pj1-B	**100%** 0G/0S	**100%** 0G/0S						
Pj1-MCU2	**100%** 0G/0S	**100%** 0G/0S	**100%** 0G/0S					
Pj1-R1	**100%** 0G/0S	**100%** 0G/0S	**100%** 0G/0S	**100%** 0G/0S				
Sf1-B	**99%** 0G/1S	**99%** 0G/1S	**99%** 0G/1S	**99%** 0G/1S	**100%** 0G/0S			
Pj3-MCU6	**97 %** 4G/9S	**97%** 4G/9S	**97%** 4G/9S	**97%** 4G/9S	**97%** 4G/10S	**100%** 0G/0S		
Pj4-A2	**97 %** 4G/9S	**97 %** 4G/9S	**97 %** 4G/9S	**97 %** 4G/9S	**97%** 4G/11S	**99%** 2G/1S	**100%** 0G/0S	
Ap1-C	**97%** 4G/10S	**97%** 4G/10S	**97%** 4G/10S	**97%** 4G/10S	**97%** 4G/9S	**99%** 2G/1S	**99%** 2G/2S	**100%** 0G/0S

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Chapter 5

Atradidymella muscivora gen. et sp. nov. (Pleosporales) and its anamorph *Phoma muscivora* sp. nov.: A new pleomorphic pathogen of boreal bryophytes.

Introduction

Bryophilous fungi are historically an understudied group (Davey and Currah 2006) and the recent description of new species from bryophyte substrates (Döbbeler and Triebel 2000, Tsuneda et al. 2000, Döbbeler 2006, Rice and Currah 2006, Davey and Currah 2007, Döbbeler 2007), as well as the taxonomically diverse suite of fungi reported from bryophyte sources (Racovitza 1959, Felix 1988, Kost 1988, Döbbeler 1997) suggest bryophytes are host to untold fungal diversity. Members of the Pleosporales are well known for their predilection for vascular plant substrates and hosts (Schoch et al. 2006) and a variety of Pleosporalean fungi have been reported from bryophytes, including representatives of the teleomorphic genera *Clathrospora* (Racovitza 1959), *Coleroa* (Henderson 1972, Fenton 1983), *Didymosphaeria*, *Leptosphaeria* (Racovitza 1959), *Massarina* (Döbbeler 1978), *Phaeosphaeria* (Möller and Dreyfuss 1996), *Pleospora*, *Protoventuria*, and *Pyrenophora* (Racovitza 1959), and the anamorphic genera *Alternaria* (Racovitza 1959, Thormann and Rice 2007), *Ascochyta* (Thormann and Rice 2007), *Phoma* (Kerry 1990, Möller and Dreyfuss 1996, Tosi et al. 2002), *Phyllosticta* (Racovitza 1959), and *Stemphylium* (Prior 1966). Given the taxonomic distribution of these genera through six different families in the order, it seems likely that the diversity

of Pleosporalean fungi with bryophyte hosts and substrates is similar to that found associated with vascular plants.

During a survey of fungi associated with common boreal bryophytes, 12 isolates of a pathogenic, pycnidium forming fungus were obtained. When the fungus was inoculated onto the model host *Funaria hygrometrica,* a teleomorph was produced with characters inconsistent with previously described pseudothecial genera. *Atradidymella muscivora* gen. et sp. nov. and *Phoma muscivora* sp. nov. are erected below on the basis of both molecular and morphological characters to accommodate the teleomorph and anamorph states of these isolates.

Materials and Methods

Isolation and teleomorph induction – Individual gametophytes of *Aulacomnium palustre*, *Hylocomium splendens*, and *Polytrichum juniperinum* from various locales in Alberta, Canada (Table 5-1) were cleaned, surface sterilized, fragmented, and plated on Mycosel (MYC: 36 g/L Mycosel Agar, Becton, Dickinson & Co., Sparks, MD, USA) or oatmeal agar (OA: 20 g/L ground oatmeal, 20 g/L agar [Invitrogen, Carlsbad, CA, USA]) amended with 0.01% oxytetracycline (Sigma, St. Louis, MO, USA) as described by Davey and Currah (2007). Isolation plates were incubated in the dark at ambient temperature and examined weekly for 6-8 weeks. Emerging hyphae were subcultured onto potato dextrose agar (PDA: 39 g/L potato dextrose agar, Difco Laboratories, Detroit, MI, USA), and OA. Isolates producing the *Phoma* anamorph of *A. muscivora* were obtained from all hosts after 3-6 weeks of incubation. The model host *Funaria hygrometrica* was cultured in vitro from spores using the methods described in Davey

and Currah (2007). Gametophytes having reached the five-leaf stage were inoculated with the ex-type strain of *A. muscivora* (UAMH 10909) by placing 5 mm diameter plugs taken from near the margin of 15 to 30 day old colonies grown on OA and placing them among the moss gametophytes. Inoculated gametophytes were incubated for 16 weeks on a 12 hour diurnal cycle with 75% relative humidity to induce the formation of the *Atradidymella* teleomorph.

Characterization – To determine growth rates and colony morphology, single point inoculations of the ex-type strain (UAMH 10909) made on PDA, OA, and malt extract agar (MEA: 20 g/L malt extract [Becton, Dickinson & Co., Sparks, MD, USA], 15 g/L agar [Invitrogen, Carlsbad, CA, USA]) were incubated in the dark at 20°C and observed every 5 d for one month. Colony measurements were calculated by averaging two perpendicular measurements of the diameter and are given as an average of 3 colonies. Mounts of the anamorph state produced on PDA and infected host moss material were made in water or polyvinyl alcohol with acid fuchsin (0.05 g acid fuchsin in 10 mL lactic acid and 1 mL glycerine mixed with 1.66 g polyvinyl alcohol dissolved in 10 mL water). Infected host mosses bearing both pycnidia of *P. muscivora* and pseudothecia of *A. muscivora* were fixed in FAA (50 mL ethanol, 5 mL acetic acid, 10 mL 40% formaldehyde, 35 mL water) for a minimum of 24 hours, subjected to an ethanol dehydration series, and embedded in paraffin wax. Sections were stained using Safranin O (Sigma, St. Louis, MO, USA), counterstained with Fast Green FCF (Sigma, St. Louis, MO, USA), and mounted using DPX mountant (Sigma, St. Louis, MO, USA). Light micrographs of all preparations were taken using an Olympus BX50 microscope with a

DP-12 digital camera. Line drawings are freehand representations of the material observed. Measurements of structures are expressed as: range (mean ± standard deviation), n=20.

For SEM, infected gametophytes were fixed in 2% glutaraldehyde (Sigma, St. Louis, MO, USA) overnight. Fixed gametophytes were rinsed in distilled water and placed in 2% tannic acid-2% guanidine hydrochloride (Sigma, St. Louis, MO, USA) solution for 4-5 hours and then postfixed overnight in 2% OsO_4 (Sigma, St. Louis, MO, USA) at 5°C. Fixed material was dehydrated in ethanol series, taken to amyl acetate, and critical-point dried in a BAL-TEC CPD 030 dryer using carbon dioxide. Dried samples were coated with gold and examined with a Hitachi S-510 scanning electron microscope at 10 or 15 kV.

Phylogenetic analyses – Isolates were grown on PDA or OA overlaid with a Cellophane™ membrane (UCB Films, Somerset, UK) for 15-30 days at ambient light and temperature. Genomic DNA was extracted as described by Davey and Currah (2007) and the internal transcribed spacer (ITS) region was amplified as described by Gibas et al. (2002) using the forward and reverse primer set ITS 4 (White et al. 1990) and BMB-CR (Lane et al. 1985). PCR amplicons were purified and sequenced as described by Davey and Currah (2007) using forward and reverse primers ITS1, ITS2, ITS3, ITS4, (White et al. 1990) and BMBC-R (Lane et al. 1985) and a consensus sequence was determined from overlapping sequence data using the software Sequencher™ (Gene Codes Corp., Ann Arbor, MI, U.S.A). Sequence identity was compared between strains using bl2seq (Tatusova and Madden 1999). A data matrix of ITS sequences including *A. muscivora,*

and members of the *Phoma-Ascochyta-Didymella* complex and representative pleosporalean families was created from sequences retrieved from GenBank. ITS sequences were aligned using MAFFT v.5.8 (Katoh et al. 2005) and the subsequent alignment manually verified. This matrix was subjected to maximum parsimony analysis using PAUP* v.4.0b10 (Swofford 2003) using Fitch parsimony, with random simple step-wise addition of taxa, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Support for branching topologies was evaluated using the parameters described above for 1000 resamplings of the data by bootstrapping analysis (Felsenstein 1985). All trees were scored for length in steps, consistency index (CI), retention index (RI), and homoplasy index (HI).

Results

Isolation and teleomorph induction – Pycnidia of *Phoma muscivora* formed within 10-20 days of plating gametophyte fragments of all three hosts on either MYC or OA. The *Atradidymella* teleomorph was not observed in culture, but formed on the model host *Funaria hygrometrica* 30-60 days after inoculation with the ex-type strain of *A. muscivora* (UAMH 10909).

Phylogenetic analysis – Pairwise identity analysis of the ITS region of isolates of *A. muscivora* revealed 100% sequence identity between isolates, and 100% similarity to two strains deposited in GenBank as *Phoma herbarum* (GenBank Accession Nos: AY337712, DQ912692). The aligned ITS data matrix consisted of a representative sequence of *Atradidymella muscivora,* sequences of *Phoma herbarum*, members of the *Phoma-Ascochyta-Didymella* clade, and representative members of the Pleosporales, was

composed of 46 taxa, and included 816 characters, of which 357 were parsimony informative. Parsimony analysis generated 21 equally parsimonious trees of 1914 steps (CI=0.511, RI=0.623, HI=0.489) whose topologies were consistent in the phylogenetic placement of *Atradidymella* relative to other species. Results of the bootstrap analysis are shown on a single most parsimonious tree (Fig. 5-1). *A. muscivora* and two GenBank isolates identified as *Phoma herbarum* (AY293803, DQ132841) form a strongly supported group (95%) nested within Clade A of the *Phoma-Ascochyta-Didymella* clade that is weakly supported (59%) as sister to the Phaeosphariaceae. Other pleosporalean genera with 1-septate, darkly pigmented ascospores (e.g. *Montagnula, Munkovalsaria, Roussoella, Venturia*) are allied to taxa outside the *Phoma-Ascochyta-Didymella* complex (Fig. 5-1).

Taxonomy

Atradidymella Davey & Currah, *gen. nov.*

MB 511986

Typus generis: Atradidymella muscivora Davey & Currah

Etymology: '*Atra*' refers to the darkly pigmented ascospores that differentiate this genus from the phylogenetically close and morphologically similar genus, *Didymella*.

Pseudothecia minuta (<200 um), fusco-brunnea, uniloculata, subglobosa ad pyriformia, setosa circum ostiolum, cum tunicis pseudoparenchymatosis. Hamathecium pseudoparenchymatosum in ascomatibus novis, quod restat in ascomatibus maturis in forma septata et filamentosa. Asci bitunicati, octo-spori, cylindrici ad clavati.

Ascosporae brunneae, fusiformes, mono-septatae, tenuiter constrictae prope septum.

Anamorphosis: Phoma

Pseudothecia minute (<200 μm), dark brown, uniloculate, subglobose to pyriform, setose around ostiole, with pseudoparenchymatous walls. Hamathecium pseudoparenchymatous in young ascomata, persisting as septate filamentous remnants in mature ascomata. Asci bitunicate, IKI-negative, 8-spored, cylindrical to clavate. Ascospores brown, fusiform, 1-septate, slightly constricted at septum. Anamorph *Phoma*.

Atradidymella muscivora Davey & Currah, *sp. nov.*

MB 511987

Etymology: '*muscivora*' refers to the pathogenic nature of the fungus on bryophyte hosts

Typus: Canada, Alberta, Wolf Lake, 78 km north of Bonnyville, Alberta, 54° 40' N 110° 57' E, on dried gametophytes of *Funaria hygrometrica* grown *in vitro* on White's medium. (UAMH 10909 – holotype)

Teleomorphosis:

Pseudothecia solitaria, a substrato erumpentia, fusco-brunnea, uniloculata, subglobosa ad elliptica vel pyriformia (58-95 um x 75-115 μm), setae breves et apicales, tunicae pseudoparenchymatosae. Hamathecium pseudoparenchymatosum in ascomatibus novis, quod restat in ascomatibus maturis in forma septata et filamentosa (1-3 μm diam). Asci cylindrici at clavati, bitunicati, octo-spori, 6-13 μm diam. Ascosporae aureo-brunneae ad fusco-brunneae, late fusiformes (4-5.5 um x 14-20 μm), rectae ad alantoidae, mono-

septatae, tenuiter constrictae prope septum, cellula superior aliquando brevior et latior quam cellula inferior.

Anamorphosis: *Phoma muscivora* M.L. Davey & R.S. Currah

MB 511988

Etymology: '*muscivora*' refers to the pathogenic nature of the fungus on bryophyte hosts

Typus: Canada, Alberta, Wolf Lake, 78 km north of Bonnyville, Alberta, 54° 40' N 110° 57' E, on dried gametophytes of *Funaria hygrometrica* grown *in vitro* on White's medium. (UAMH 10909 – holotype, UAMH 10910,10911 - paratypes)

Pycnidia solitaria vel in glomeribus, a substrato erumpentia, globosa ad subglobosa, collum breve, (70-212.5 x 85-150 μm), alba cum collo fusco, pycnidium totum fuscum quando maturant. Conidia elliptica, unicellularia (1-2 x 2.5-5 μm).

On leaves, stems, and rhizoids of *Funaria*, causing chlorosis and often death (Figs. 5-2, 5-8, 5-9). Hyphae hyaline and frequently penetrating host tissues, forming intracellular clumps that become dematiaceous and mature to form pycnidia or pseudothecia that are erumpent 10-60 d post inoculation (Figs. 5-2, 5-8, 5-9). On PDA, young (7 d) colonies dense, floccose with funiculate projections, often forming sectors of variously pigmented sterile and fertile mycelia. Young colonies white, cream, pale orange, or pale pink, producing pink or red pigments visible in reverse that turn blue with the addition of 1M NaOH, mature (>10 d) colonies often darkening to grey or grey-brown. Colonies attaining 33 mm diameter after 7 d at 20°C and producing pycnidia after 7-20 d, predominantly on funiculate projections (Fig. 5-7). On MEA, young

colonies white, cream, or pale orange, with sparse white aerial hyphae, and a pale margin of submersed hyphae, reverse white. Colony darkening with age to grey-brown with dark brown reverse, becoming floccose with funiculate projections, and attaining 35 mm after 7 d at 20°C (Fig. 5-7). Pycnidia sparse, not immersed in media, forming after 15-30 d. On OA, young colonies dense, floccose to velvety with funiculate projections, white, cream, pale orange or pale pink, and producing pink-orange or red pigments visible in reverse. Maturing to off-white or pale grey-brown with dark brown reverse, attaining 37 mm after 7 d at 20°C, producing pycnidia after 10-20 d (Fig. 5-7).

Teleomorph – Pseudothecia solitary, erumpent from underlying host cell (Figs. 5-3, 5-12), dark brown, uniloculate, subglobose to elliptic or pyriform (58-95 μm x 75-115 μm) with short concolorous, occasionally septate setae around ostiole (Figs 5-3, 5-11-5-12). Peridium approximately 10 μm wide with 3 layers of pseudoparenchymatous cells, the outermost with darkly pigmented walls (Figs. 5-3, 5-11). Pre-ascogenous centrum of hyaline pseudoparenchymatous elements and darkly pigmented, branched, septate filamentous elements (Fig. 5-10). Hamathecium pseudoparenchymatous in young ascomata (Fig. 5-11), persisting as septate filamentous remnants (1-3 μm in diameter) in mature ascomata (Fig. 5-12). Crozier formation was not observed. Asci cylindrical to clavate, bitunicate, 8-spored, IKI negative, 6-13 μm in diameter, grouped in a small fascicle of 10-20 at base of pseudothecium (Figs. 5-3-5-4). Ascospores arranged bi- or tri-seriately in ascus (Fig. 5-4, 5-11), golden brown to dark brown, broadly fusiform, 4-5.5 (4.5 $\pm$ 0.6) x 14-20 (16.8 $\pm$ 1.5) μm, smooth, straight to allantoid, 1-septate, slightly

constricted at septum, the upper cell sometimes shorter and broader than the lower (Figs. 5-4, 5-13, 5-17).

Anamorph – Pycnidia solitary or in clusters, erumpent from substrate (Figs. 5-8, 5-9), globose to subglobose with short neck (Fig. 5-5), 70-212.5 (121.1 ± 34.4) x 85-250 (140.6 ± 39.5) µm. Young pycnidia white with dark neck, entire pycnidium becoming dark with age. Pycnidium wall 3-9 µm thick, consisting of 3-4 layers of flattened pseudoparenchymatous cells, the outermost becoming darkened and compressed with age (Fig. 5-14). Phialides hyaline, broad ampulliform, mostly wider than tall (Figs. 5-6, 5-15), 3-7 (4.5 ± 1.1) x 3-5 (3.9 ± 0.7) µm. Conidia hyaline, elliptic, smooth, unicellular (Fig. 5-16), 1-2 (1.2 ± 0.3) x 2.5-5 (3.3 ± 0.5) µm, exuded as a slimy cream, pale yellow, or pale pink droplet.

Additional specimens examined – Canada: Alberta, Jackfish Lake Bog, 7 km east of Jackfish Lake along HWY 2, isolated from gametophytes of *Aulacomnium palustre*, M.L. Davey (Ap1-C, Ap1-S, Ap1-R); Alberta, Redwater Recreation Area, 12 km east of Redwater, isolated from gametophytes of *Hylocomium splendens*, M.L. Davey, (UAMH 10910, Hs7-D, Hs7-J); Alberta, Redwater Recreation Area, 12 km east of Redwater, isolated from gametophytes of *Polytrichum juniperinum,* M.L. Davey, (UAMH 10911, Pj9-A); Alberta, Wolf Lake, 78 km north of Bonnyville, isolated from gametophytes of *Polytrichum juniperinum,* M.L. Davey, [UAMH 10909 (ex-type specimen), Pj8-C, Pj8-G].

Discussion

Among previously established pseudothecial genera with reduced stromata, darkly pigmented pseudothecia, and fusiform, 1-septate ascospores, *Atradidymella* is morphologically similar to *Coleroa*, *Venturia, Didymella*, *Didymosphaeria*, and *Roussoëlla,* of which only *Coleroa* (Henderson 1972, Fenton 1983) and *Didymosphaeria* (Racovitza 1959) have previously been reported from bryophytes. However, *Atradidymella* can be distinguished from these genera on the basis of ascospore and pseudothecium morphology, and stroma ontogeny. The smooth, darkly pigmented ascospores of *Atradidymella* distinguish it from the hyaline ascospores of *Didymella* (Corbaz 1957) and the reticulately or striately ornamented ascospores of *Roussoëlla* (Aptroot 1995b), while its minute pseudothecia and pseudoparenchymatous peridium distinguish it from *Didymosphaeria,* which has larger pseudothecia and a hyphal peridium of *textura intricata* (Aptroot 1995a). *Atradidymella* produces an extremely reduced stroma within a single host cell, from which the pseudothecium ultimately emerges. This limited stroma formation distinguishes *Atradidymella* from *Coleroa* and *Venturia*, both of which form more extensive stromata that originate both inter- and intra-cellularly in the epidermal and sub-epidermal regions of vascular plant leaves and stems (Barr 1968).

Parsimony analysis of the ITS region supports morphological data suggesting *Atradidymella muscivora* represents both a new species and genus. The only teleomorphic genera with fusiform, pigmented, 1-septate ascospores that have been reported with *Phoma* or *Ascochyta* anamorphs are *Didymosphaeria* (Farr et al. 1989),

Massarina (de Hoog 1979), and *Otthia* (Grove 1935), all of which belong to families that are phylogenetically distant from *Atradidymella*. Although nested within the *Phoma-Ascochyta-Didymella* clade and resembling *Didymella* in hamathecium development, and ascospore and pseudothecium shape and size, *Atradidymella* cannot be accommodated within *Didymella* on account of its pigmented ascospores and intracellular stroma formation (Corbaz 1957, Corlett 1981, Skarshaug 1981, de Neergaard 1989).

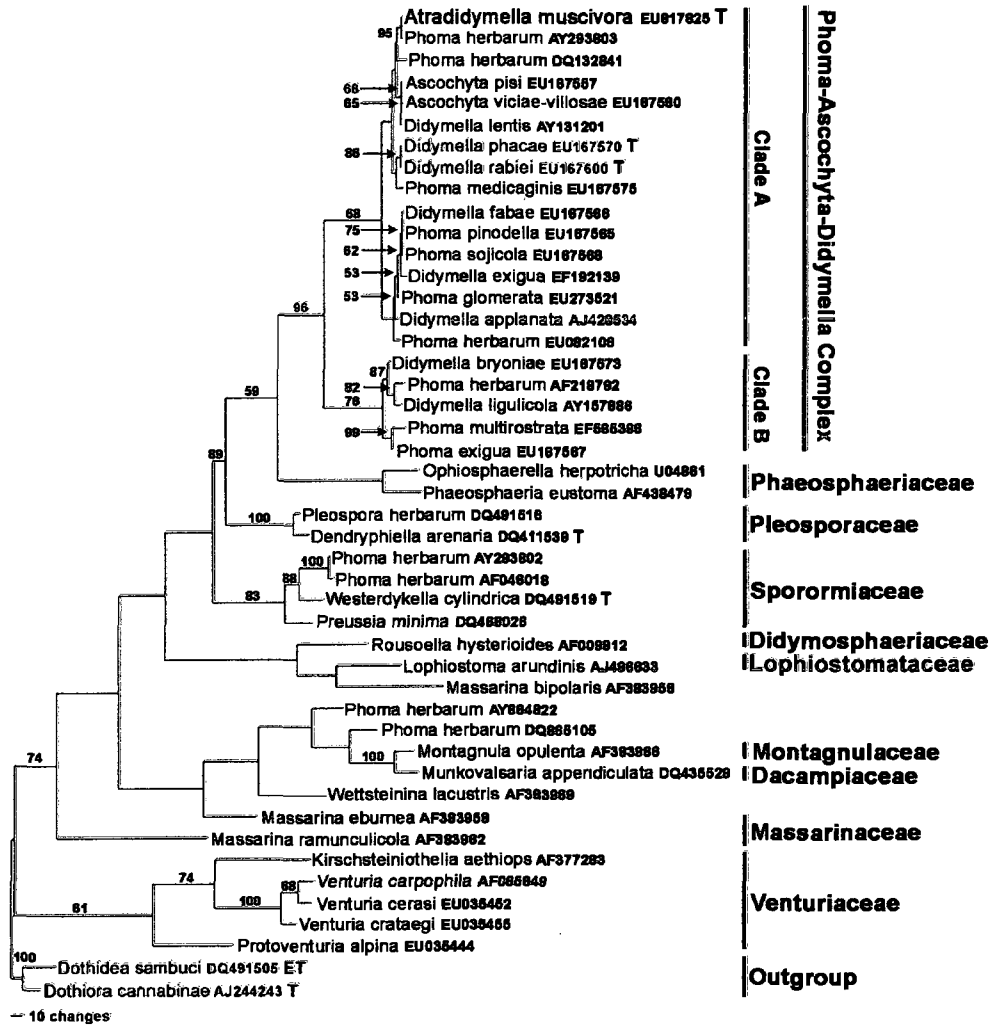
Isolates of *Atradidymella muscivora* have a high degree of ITS sequence similarity (99-100%) to four sequences deposited in GenBank as *Phoma herbarum* (GenBank Accession Nos: AY337712, AY293803, DQ132841, DQ912692) that were isolated independently from a variety of synthetic and organic substrates. However, sequence data is not available for the type specimen of *P. herbarum*, and phylogenetic analysis of available sequences for the species (Fig. 5-1), and analysis of its physiological and morphological characters (Montel et al. 1991) suggest the current species concept is polyphyletic. The *Phoma* anamorph of *Atradidymella muscivora* is also morphologically most similar to *P. herbarum* with its pink to yellow or white spore masses; unicellular, ellipsoid to cylindric conidia; ampulliform phialides; and pycnidia that are initially pale brown with a dark ostiolar neck, and later darken with age. However, *P. muscivora* is morphologically differentiated from *P. herbarum* by its slightly smaller conidia (1-2 x 2.5-5 μm versus 1.5-3 x 3.5-8 μm) (Boerema 1964, Boerema 2004).

Despite the potential for taxonomic confusion between *P. herbarum* and *P. muscivora*, the *Phoma* anamorph of *Atradidymella* was formally named because it is the predominantly visible state in the disease cycle of *A. muscivora*, is the morph most easily

induced to sporulate in vitro, and can be morphologically distinguished from *P. herbarum* based on conidium size. Given that *P. muscivora* is morphologically very similar to *P. herbarum*, it is possible that those isolates with high ITS similarity to *P. muscivora* could also be accommodated within this species upon verification of conidium size. *Phoma herbarum* has previously been reported from mosses (Kerry 1990, Tosi et al. 2002) and some of these may also represent further records of *P. muscivora* from bryophyte hosts.

Atradidymella muscivora represents new diversity within the bryophilous Pleosporales. The isolates described herein are the first report of a teleomorph allied to the *Phoma-Ascochyta-Didymella* clade from a bryophyte host and only the second description of a Pleosporalean pathogen actively causing chlorosis in its host, after *Coleroa turfosorum* (Fenton 1983). Finally, the extreme stroma reduction and localization to a single host cell observed in *A. muscivora* has not been previously reported among pleosporalean bryophilous fungi and may represent a unique specialization to a bryophyte habit.

Figure 5-1. One of 21 equally parsimonious trees (1914 steps, CI=0.511, RI=0.623, HI=0.489) inferred from a maximum parsimony analysis of ITS sequences showing the placement of *Atradidymella muscivora* (including its anamorph, *P. muscivora*) among members of the Pleosporales. Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. GenBank accession numbers are given following species names. Ex-type sequences are indicated with a 'T' following the accession number. 'ET' indicates ex-epitype.


Atradidymella muscivora EU617625 T
Phoma herbarum AY293603
Phoma herbarum DQ132841
Ascochyta pisi EU167567
Ascochyta viciae-villosae EU167580
Didymella lentis AY131201
Didymella phacae EU167570 T
Didymella rabiei EU167600 T
Phoma medicaginis EU167575
Didymella fabae EU167566
Phoma pinodella EU167565
Phoma sojicola EU167568
Didymella exigua EF192139
Phoma glomerata EU273521
Didymella applanata AJ426534
Phoma herbarum EU082108
Didymella bryoniae EU167573
Phoma herbarum AF218762
Didymella ligulicola AY157686
Phoma multirostrata EF585386
Phoma exigua EU167567
Ophiosphaerella herpotricha U04861
Phaeosphaeria eustoma AF438479
Pleospora herbarum DQ491516
Dendryphiella arenaria DQ411539 T
Phoma herbarum AY293602
Phoma herbarum AF046018
Westerdykella cylindrica DQ491519 T
Preussia minima DQ468026
Rousoella hysterioides AF009812
Lophiostoma arundinis AJ496633
Massarina bipolaris AF383956
Phoma herbarum AY864822
Phoma herbarum DQ885105
Montagnula opulenta AF383986
Munkovalsaria appendiculata DQ435529
Wettsteinina lacustris AF383989
Massarina eburnea AF383958
Massarina ramunculicola AF383962
Kirschsteiniothelia aethiops AF377283
Venturia carpophila AF065649
Venturia cerasi EU035452
Venturia crataegi EU035455
Protoventuria alpina EU035444
Dothidea sambuci DQ491505 ET
Dothiora cannabinae AJ244243 T
10 changes
Clade A
Clade B
Phoma-Ascochyta-Didymella Complex
Phaeosphaeriaceae
Pleosporaceae
Sporormiaceae
Didymosphaeriaceae
Lophiostomataceae
Montagnulaceae
Dacampiaceae
Massarinaceae
Venturiaceae
Outgroup

Figures 5-2 to 5-6. Line drawings of *Atradidymella muscivora* and its anamorph, *Phoma muscivora*.

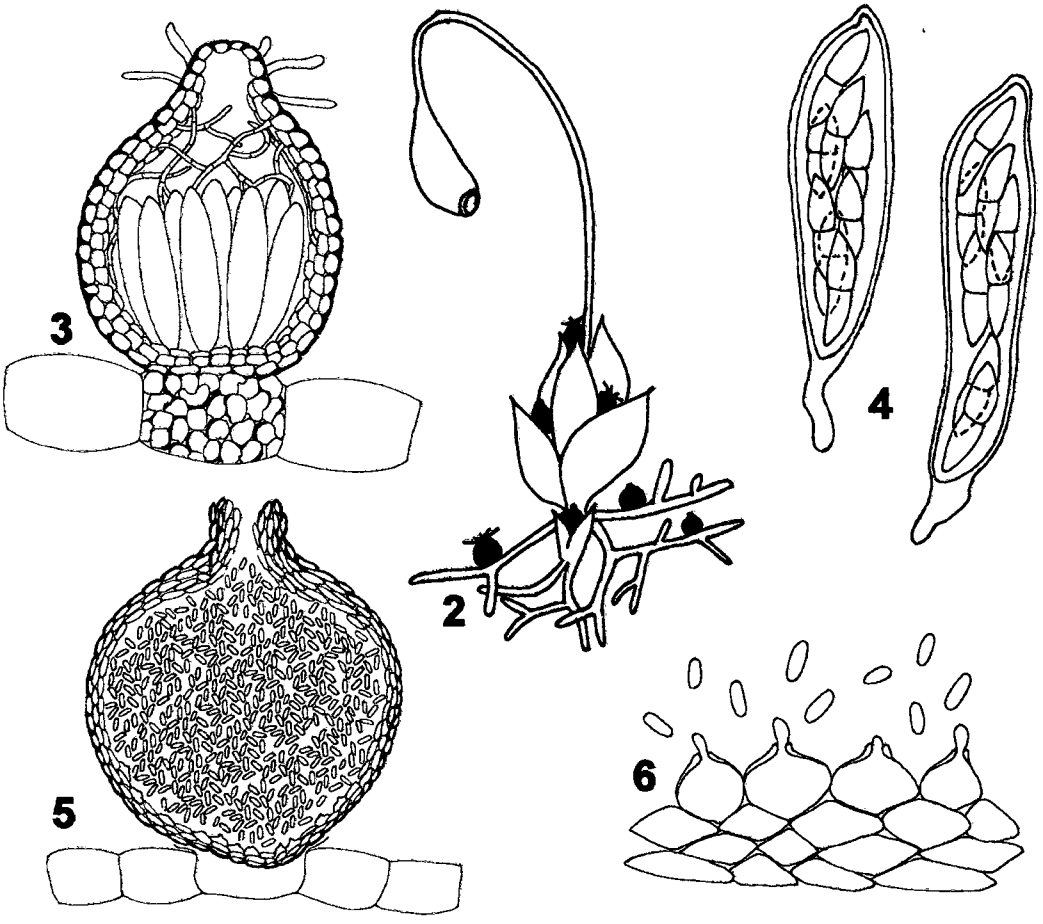
Figure 5-2. *Funaria hygrometrica* plant bearing both pseudothecia and pycnidia on leaves and rhizoids of gametophyte.

Figure 5-3. Pseudothecium and stroma of *A. muscivora* on a moss leaf.

Figure 5-4. Asci and ascospores of *A. muscivora*.

Figure 5-5. Pycnidium of *P. muscivora* on a moss leaf.

Figure 5-6. Phialides and conidia of *P. muscivora*.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Figures 5-7 to 5-17. *Atradidymella muscivora* and its anamorph, *Phoma muscivora.* (Figs. 5.7, 5.10-5.13 = UAMH 10909; Figs. 5.8, 5.14-5.17 = UAMH 10911; Fig. 5.9 =UAMH ##Ap1-S; Figs. 5.10-5.12, 5.14-5.15 = Paraffin sections stained with Safranin O-Fast green FCF; Fig. 5.13 = wet mount; Figs. 5.16-5.17 = SEM)

Figure 5-7. Composite figure of colonies of the ex-type strain of *Atradidymella muscivora* (UAMH 10909) after 10d incubation at 20°C. Clockwise from top left: PDA, OA, MEA. Scale bar = 15 mm.

Figure 5-8. *Funaria hygrometrica* gametophyte infected with *P. muscivora* (UAMH 10911). Two pycnidia (arrowheads) are erumpent from the leaf surface. Scale bar = 1 mm.

Figure 5-9. Stem of *Funaria hygrometrica* inoculated with *P. muscivora* (UAMH Ap1-S) showing emerging pycnidia. Scale bar = 150 μm.

Figure 5-10. Cross section of immature pseudothecium containing both hyaline and dematiaceous (arrowhead) stromatal elements. Scale bar = 25 μm.

Figure 5-11. Longitudinal section of a young pseudothecium with intact asci and pseudoparenchymatous hamathecial elements. Scale bar = 25 μm.

Figure 5-12. Longitudinal section of a mature pseudothecium of *A. muscivora*. Asci have deliquesced, leaving a jumbled mass of ascospores within the pseudothecium, and hamathecium consists of septate, filamentous remnants (arrowhead). The stroma of the pseudothecium is restricted to a single gametophyte cell (bottom left). Scale bar = 20 μm.

Figure 5-13. Composite figure showing variation in ascospore morphology between pseudothecia of the ex-type strain.

Figure 5-14. Longitudinal section of a pycnidium of *P. muscivora*. Note the neck has darkened, while the remainder of the pycnidium walls are compressed, but have yet to darken. Scale bar = 40 μm.

Figure 5-15. Longitudinal section of a pycnidium showing conidia produced by stout, ampulliform phialides (arrowheads). Scale bar = 15 μm.

Figure 5-16. Scanning electron micrograph of the smooth, ellipsoid to short cylindric conidia of *P. muscivora*. Scale bar = 5 μm.

Figure 5-17. Smooth, broad fusiform, 1-septate ascospores of *A. muscivora*. Arrowhead indicates the slight constriction at the septum of an ascospore. Scale bar = 10 μm.

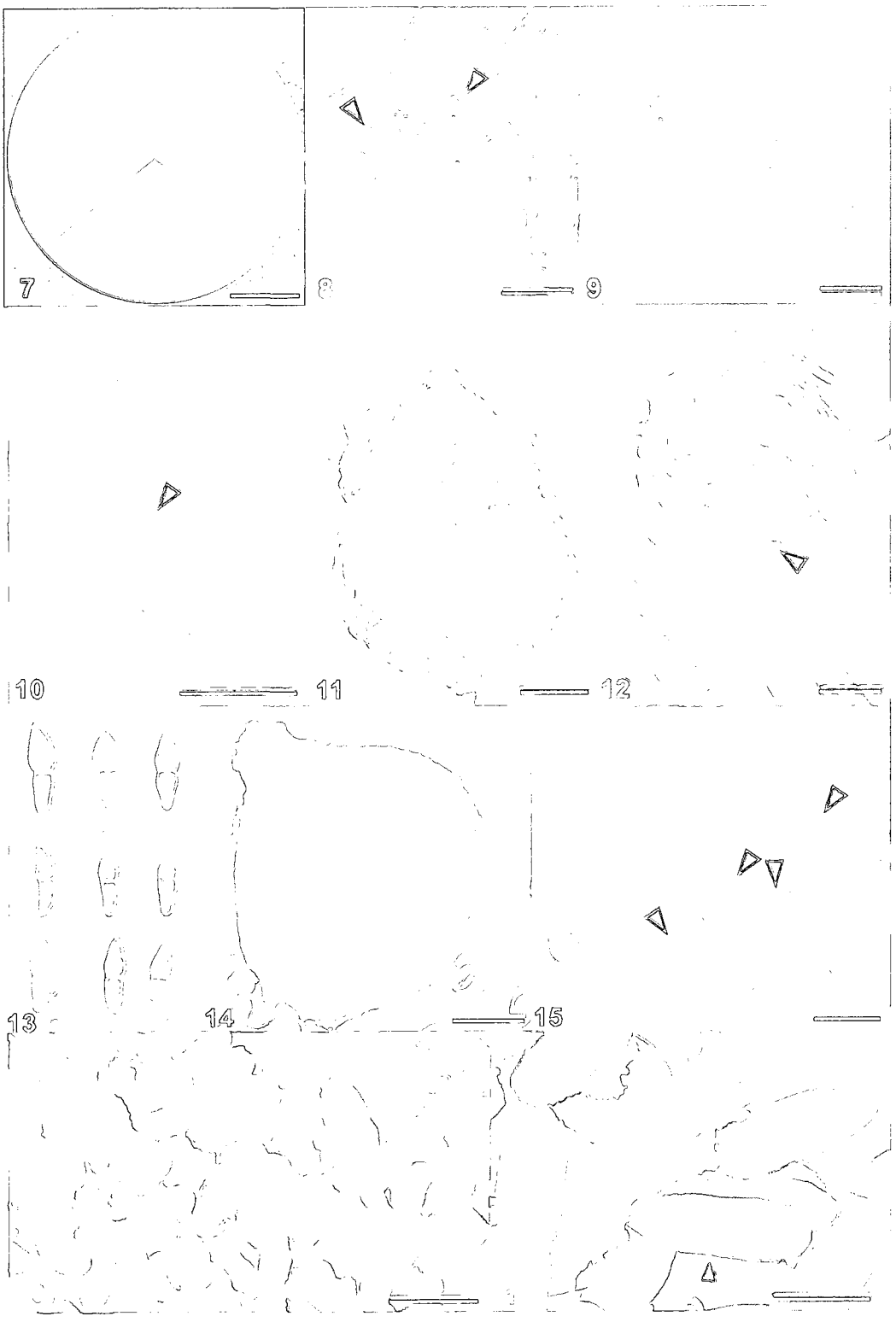
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Table 5-1. Strains of *Atradidymella muscivora* isolated from the boreal mosses *Aulacomnium palustre, Hylocomium splendens*, and *Polytrichum juniperinum* in Alberta, Canada.

Site No.	Location	Site Description	Isolate	Host	GenBank #	UAMH #
1	**Jackfish Lake Bog** Along HWY 2, 7km east of Jackfish Lake	Mixed *Sphagnum* bog with an overstory of *Picea mariana*	Ap1-Q	*A. palustre*	EU817826	-
			Ap1-R	*A. palustre*	-	-
			Ap1-S	*A. palustre*	EU817827 EU817830	-
2	**Redwater Natural Area** NE 20-57-20 W4	Mixed *Sphagnum* wetlands with an overstory of *Picea mariana* and *Larix laricina* and uplands transitioning to *Pinus banksiana* dominated dunes	Hs7-C	*H. splendens*	EU817828	UAMH 10910
			Hs7-D	*H. splendens*	-	-
			Hs7-J	*H. splendens*	-	-
			Pj9-A	*P. juniperinum*	-	-
			Pj9-C	*P. juniperinum*	EU817829	UAMH 10911
3	**Wolf Lake** NW 35-65-7 W4 54° 40' N 110° 57' E	Wet lakeshore with an overstory of *Picea glauca, Betula papyrifera,* and *Salix* sp.	Pj8-C	*P. juniperinum*	-	-
			Pj8-D^T	*P. juniperinum*	EU817825	UAMH 10909
			Pj8-G	*P. juniperinum*	-	-

Note: The symbol ^{T} indicates the isolate is ex-type.

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Chapter 6

The disease etiology of the fungus *Atradidymella muscivora* on its bryophyte hosts.

Introduction

Fungal pathogens of mosses have been reported since the late 19th century (Racovitza 1959), and their general symptoms of host chlorosis and necrosis are well documented (Wilson 1951, Hawksworth 1973, Redhead 1981, Tsuneda et al. 2001a, Döbbeler 2003). Great diversity in host cell penetration and disruption, mechanisms of obtaining nutrients, host responses, and disease etiology has been observed among bryophyte pathogens (Döbbeler 1997, Davey and Currah 2006) and many species exhibit a high degree of specialization to their bryophilous habit (Döbbeler 1997). However, comprehensive studies of disease etiology have been conducted for only a limited number of bryophilous pathogens. Of these, most are basidiomycetes, including *Eocronartium muscicola* (Pers.) Fitzp. (Boehm and McLaughlin 1988), *Arrhenia retiruga* (Bull.) Redhead (Hassel and Kost 1998), and *Tephrocybe palustris* (Peck) Donk (Redhead 1981, Untiedt and Müller 1985). Although ascomycete pathogens of bryophytes have been reported and symptoms of infection are often well documented (Racovitza 1959), only the disease etiology of *Scleroconidioma sphagnicola* Tsuneda, Currah & Thormann (Tsuneda et al. 2001a) has been characterized thoroughly using modern microscopic techniques.

Atradidymella muscivora Davey & Currah is a pathogen of mosses that produces a *Phoma* anamorph (*P. muscivora* Davey & Currah), and has phylogenetic affinities to

the *Phoma-Ascochyta-Didymella* complex that is sister to the Phaeosphariaceae in the Pleosporales (M.L. Davey & R.S. Currah, University of Alberta, unpublished manuscript). Members of this complex are well known as saprophytes, parasites, and pathogens of vascular plants (Corlett 1981, Schoch et al. 2006) and species of *Phoma, Ascochyta,* and *Didymella* have previously been reported from bryophytes and hepatophytes (Racovitza 1959, Döbbeler 1978, Kerry 1990, Möller and Dreyfuss 1996, Tosi et al. 2002, Thormann and Rice 2007). Using light and scanning electron microscopy, and extracellular enzyme profiling, we characterized in detail the disease etiology of *A. muscivora* infecting the model host *Funaria hygrometrica* Hedw. and made observations of host-fungus interactions between *A. muscivora* and its natural hosts.

Materials and Methods

Host-fungus interactions – The model host *Funaria hygrometrica,* and native hosts *Polytrichum juniperinum* Hedw. and *Hylocomium splendens* (Hedw.) Schimp. were cultured *in vitro* from spores using the methods described in Davey and Currah (2007). Another native host, *Aulacomnium palustre* (Hedw.) Schwaegr. was cultured in vitro from gemmae that were surface sterilized in 1 mL of 1% NaOCl for 3 minutes with agitation by vortexing, and then washed three times in 1 mL of sterile distilled water for one minute. The surface sterilized gemmae were suspended in sterile distilled water and cultured in the same manner as described for the spores above. Gametophytes of the five-leaf stage were inoculated with isolates of *Atradidymella muscivora* (UAMH [ap1-q/ap1-s], UAMH 10909, 10910, 10911) by placing 5-mm-diameter plugs taken from near the margin of 15 to 30 day old colonies grown on oatmeal agar (OA: 20 g/L agar

[Invitrogen, Carlsbad, CA, USA], 20 g/L ground oatmeal) and placing them among the moss gametophytes. The inoculated gametophytes, and uninoculated control gametophytes were incubated for 12 weeks and observed by light microscopy (LM) weekly. Important stages in the disease etiology were selected for further examination by LM and scanning electron microscopy (SEM).

Microscopy – Health of gametophytes was examined by a stereomicroscope and by mounting individual leaves or entire gametophytes in water or polyvinyl alcohol with acid fuchsin (0.05g acid fuchsin in 10mL lactic acid and 1mL glycerine mixed with 1.66g polyvinyl alcohol dissolved in 10mL water) and examining them under low power with a compound microscope. Infected host tissues were fixed in FAA (50 mL ethanol, 5 mL acetic acid, 10 mL 40% formaldehyde, 35 mL water) or 2% glutaraldehyde (Sigma, St. Louis, MO, USA) for a minimum of 24 hours, dehydrated in an ethanol series, and embedded in paraffin wax. Sections were stained using Safranin O (Sigma, St. Louis, MO, USA), counterstained with Fast Green FCF (Sigma, St. Louis, MO, USA), and mounted using DPX mountant (Sigma, St. Louis, MO, USA). Light micrographs of all preparations were taken using an Olympus BX50 microscope with a DP-12 digital camera.

For SEM, infected gametophytes were fixed in 2% glutaraldehyde (Sigma, St. Louis, MO, USA) overnight. Fixed gametophytes were rinsed in distilled water and placed in 2% tannic acid-2% guanidine hydrochloride (Sigma, St. Louis, MO, USA) solution for 4-5 hours and then postfixed overnight in 2% OsO_4 (Sigma, St. Louis, MO, USA) at 5°C. Fixed material was dehydrated in ethanol series, taken to amyl acetate, and

critical-point dried in a BAL-TEC CPD 030 dryer using carbon dioxide. Dried samples were coated with gold and examined with a Hitachi S-510 scanning electron microscope at 10 or 15 kV.

Enzyme characterization – Three strains of *Atradidymella muscivora* (UAMH Ap1-Q, 10909, 10911) were tested for amylase, gelatinase, pectinase, cellulase, lipase, and insoluble and soluble polyphenolic oxidase enzyme activities using indicator media, as described by Davey & Currah (2007).

Results

Preliminary LM examinations of *A. muscivora* infecting all four host species indicated virulence and host responses were similar among the native hosts and model host. Therefore, detailed etiological characterization was limited to *Funaria hygrometrica*.

Infection of Funaria hygrometrica – Initial stages of infection (1-10 days) by *A. muscivora* were characterized by the production of floccose, white aerial mycelium on the surface of the gametophytes followed by attempted penetration of rhizoids, leaves, and protonemata. Ingress into the host was either by direct penetration of cell walls by vegetative hyphae (Fig. 6-1) or by the formation of appressoria and penetration pegs (Figs. 6-2, 6-3). Thickened, pigmented, papilla-like deposits surrounded penetration sites and the intruding hyphae (Figs. 6-3, 6-4). These papilla-like deposits were simple, bifurcating or stellate depending on the branching pattern of the intruding hypha. Hyaline intracellular hyphae, 1-2.5 μm in diameter, were observed after 5-20 days (Fig. 6-5, 6-7), and the infected leaf and rhizoid cells contained few or no chloroplasts (Figs. 6-

6). Although hyphae exhibited intracellular growth, penetration of a new cell frequently elicited a host response. Eventually, leaves of infected gametophytes became chlorotic and mottled with dark brown spots where the host response had occurred (Figs. 6-8, 6-9). Cell wall degradation was limited to areas invaded by hyphae of *A. muscivora,* and was evidenced by hyphae growing between the lamellate layers of the host cell wall and by general thinning of the cell wall that resulted in the formation of localized voids (Figs. 6-10 to 6-12).

Pycnidia formed on the gametophyte surface after 7-20 days and were initiated primarily within leaf and stem tissues, and less frequently within rhizoids. Pycnidial initials were produced intracellularly, each originating as a tightly packed, dematiaceous, hyphal proliferation within a single cell of a leaf, rhizoid, or the stem epidermis (Fig. 6-13). Subsequent enlargement of the hyphal mass caused the cell to rupture and the developing pycnidium enlarged at the surface of the gametophyte (Figs. 6-14 to 6-18). Pseudothecium development was analogous to pycnidium development, with stroma initials being produced within a single cell that was ultimately ruptured by the developing pseudothecium (Figs. 6-20 to 6-22). Under dry conditions, conidia were exuded in slimy droplets from the pycnidia, while under wet conditions, conidia were exuded in a cirrhus within the surrounding water droplet (Fig. 6-19). Active release of ascospores from pseudothecia was not observed, and ascospores were left free within the pseudothecium (Fig. 6-22) after the ascus walls deliquesced.

Infection of natural hosts – Initial stages of infection of *H. splendens* and *Aul. palustre* by *A. muscivora* were identical to those in *F. hygrometrica*. Host penetration

occurred directly or through the formation of appressoria (Fig. 6-23), and both hosts responded by depositing layers of darkly pigmented material around penetration sites and invading hyphae (Fig. 6-24). As observed in *F. hygrometrica*, hyphae grew intracellularly, and penetration of a new cell frequently elicited a host response (Fig. 6-28). Production of pycnidia within single host cells was consistent between *Funaria, Aulacomnium,* and *Polytrichum* (Figs. 6-25, 6-29). However, in *Aulacomnium*, pycnidia formed primarily in cells of gemmae and at the gemma axils (Figs. 6-26 to 6-29). In *Polytrichum,* pycnidia frequently formed in the photosynthetic lamellae cells of the leaves or in the protective, hyaline, cells of the leaf margin. Cell wall degradation in all three natural hosts was similar to that observed in the *A. muscivora* - *F. hygrometrica* model system (Figs. 6-30, 6-31).

Enzyme activity and cell wall degradation – All isolates tested negatively for amylase, pectinase, lipase, and insoluble polyphenolic oxidase activities. Tests for cellulase, gelatinase, and soluble polyphenolic oxidase activities were positive in all isolates.

Discussion

Atradidymella muscivora causes chlorosis, necrosis, and sometimes death of its hosts, indicating it is a pathogen of mosses. The fungus appears to be a generalist pathogen with a broad host range among temperate mosses, as it has been isolated from three different genera representing three bryophyte orders (Polytrichales, Hypnales,

Bryales) (M.L. Davey and R.S. Currah, University of Alberta, unpublished manuscript) and also readily infects the model host *F. hygrometrica* (Funariales).

The disease cycle of *A. muscivora* is initiated with host penetration, with or without the formation of appressoria. Positive tests for cellulase and soluble polyphenolic oxidase activity indicate that penetration probably involves some degree of lytic dissolution of the host cell wall. *Funaria hygrometrica, Aul. palustre*, and *H. splendens* respond to the intruding hyphae by depositing layers of darkly pigmented material around them, ultimately forming a structure resembling the callose- and polyphenolic-rich papillae formed by vascular plants in response to penetration attempts by parasitic and pathogenic fungi (Aist and Bushnell 1991, Agrios 1997). Bifurcating and stellate papillae indicate that the fungus may be responding to initial host defenses by branching in an attempt to avoid or escape from the papilla. Papilla formation has previously been reported in *Funaria hygrometrica* (Martinez-Abaigar et al. 2005) and a taxonomically diverse suite of mosses infected by basidiomycetes and ascomycetes (Racovitza 1959, Hassel and Kost 1998). Similar responses have been described in both hepatophytes (Racovitza 1959, Read et al. 2000) and angiosperms (Aist and Bushnell 1991, Agrios 1997), suggesting this response to be a generalized, early-evolved resistance mechanism that is common to all plants, similar to the salicylic-acid-induced defense responses that have been described in the moss *Physcomitrella patens* and a wide variety of vascular plants (Andersson et al. 2005).

Host cells that have been successfully penetrated by *A. muscivora* lack chloroplasts. It is unknown whether *A. muscivora* produces a chloroplast-degrading

phytotoxin, but other members of the Phaeosphaeriaceae and *Phoma-Ascochyta-Didymella* clade to which *A. muscivora* is evolutionarily close (M.L. Davey and R.S. Currah, University of Alberta, unpublished manuscript) are known to produce a variety of phytotoxins (Pedros and Chumala 2005, Vikrant et al. 2006), including calmodulin-inhibiting enolides that can interfere with photosynthesis (Rivero-Cruz et al. 2003). The patterns of cell wall degradation and the ability of *A. muscivora* to degrade both cellulosic and polyphenolic cell wall components suggest that the fungus does not obtain its nutritients solely from the cytoplasmic contents of its host, and can also function as an opportunistic saprobe on dead material. The generalized thinning and formation of localized pits that appear in gametophytes infected with *A. muscivora* have previously been observed in the degradation of *Sphagnum fuscum* by *Oidiodendron maius* (Tsuneda et al. 2001b), but represent the first instance in which this behaviour has been documented in a bryophyte pathogen.

After successful host penetration and colonization, pycnidia and pseudothecia of *A. muscivora* are initiated, each within a single cell, and both structures subsequently become erumpent from the underlying host cell. The fungus' lack of pectinolytic enzymes to aid in intercellular growth via the breakdown of the middle lamella, in conjunction with the initiation of a host response each time a cell is penetrated likely increases the energetic cost of forming a large stroma involving multiple host cells. This may have created evolutionary pressure for stroma reduction and the compartmentalization of the reproductive lifecycle stages of *A. muscivora* into single host cells. Highly reduced reproductive structures that are limited to a single host cell have

been reported among other bryophilous and hepaticolous fungi with various taxonomic affinities (Racovitza 1959, Döbbeler 1980), suggesting that such reduction allows for specialization to a bryophyte host, and is a character that has multiple origins among bryophilous fungi.

The ability of *A. muscivora* to produce rapidly a sporulating asexual state is likely integral to its persistence on a moss host, because many boreal species, including *Hylocomium splendens* and *Polytrichum juniperinum,* are poikilohydric and frequently dry out, becoming osmotically inhospitable and having little metabolic activity (Proctor and Tuba 2002). The rapid production of pycnidia would allow *A. muscivora* to exploit periods of wet weather for dispersal and colonization of new hosts, effectively perpetuating the disease cycle. Conidia formed in a slimy droplet are most conducive to dispersal by water. This may be adaptive by ensuring conidia dispersal occurs during periods suitable for germination, thus maximizing potential for infection. The lack of active spore dispersal and retention of most ascospores in the pseudothecium may indicate that the entire ascoma of *A. muscivora* functions as a diaspore, or that the teleomorph functions primarily to introduce and maintain genetic variability and dispersal of the ascospores is dependent on mechanical disruption of the ascocarp.

Atradidymella muscivora exhibits some microniche specialization on its native hosts, forming pycnidia preferentially on the leaf lamellae and along the involute leaf margins of *Pol. juniperinum,* and on or in the axils of gemmae in *Aul. palustre*. In *Pol. juniperinum*, the relatively large quantities of photosynthate produced by the lamellae and the narrow spaces between them would provide ample resources and a sheltered location

for pycnidium production. The nutrient-rich gemmae of *Aul. palustre*, which are modified leaves, present a similar rich microniche for the formation of fungal reproductive structures. In this position, the incipient fungal sporocarps ostensibly intercept and absorb nutrients that would otherwise go to the formation of the host's vegetative propagules. This strategy is comparable to the bryophilous basidiomycete *Eocronartium muscicola* which parasitizes gametophyte transfer cells, intercepting nutrients destined for sporophyte production and diverting them to the formation of the parasite's basidiomata (Boehm and McLaughlin 1988). A similar co-opting of host resources may be occurring in the ascomycete *Lizonia baldinii* which preferentially colonizes the reproductive apices of its host's gametophytes (Racovitza 1959, Döbbeler 2003).

Although *A. muscivora* exhibits many structural features common to plant pathogenic fungi, its disease etiology is unique when compared to previously characterized fungal pathogens of bryophytes. Both ascomycete and basidiomycete pathogens have been reported to penetrate bryophyte hosts directly, or via appressoria (Racovitza 1959, Kost 1988, Döbbeler 1997), although *A. muscivora* represents the first report of concurrent lytic and mechanical penetration. Papilla induction in host cells has been observed in other bryophilous ascomycetes, including *Nectria muscivora* and *Teichospora jungermannicola* (Racovitza 1959), and basidiomycetes such as *Galerina paludosa* (Redhead 1981), *Arrhenia retiruga* (Hassel and Kost 1998) and *Rickinella fibula* (Kost 1988). However, in these examples, the host response is associated with

primary penetration attempts rather than with intracellular hyphal growth, as observed in *A. muscivora*.

In summary, *A. muscivora* is a generalist pathogen of mosses, whose life cycle, morphology, and patterns of host and microniche exploitation all show specific adaptations to the bryophyte host. Further detailed studies of pathogen-host interactions among an untold diversity of bryophilous fungi and their hosts is expected to illustrate consistent etiologic patterns among fungal plant pathogens along with a wealth of unique adaptive strategies.

Figures 6-1 to 6-12. Colonization and degradation of *Funaria hygrometrica* by *Atradidymella muscivora* (Figs 6.1-6.2, 6.6, 6.8 = UAMH 10911; Figs. 6.5, 6.7, 6.9-6.12 = UAMH 10909, Figs. 6.3-6.4 = Ap1-Q; Figs. 6.1-6.2, 6.5, 6.7, 6.10-6.12 = SEM; Figs. 6.3-6.4 = lactofuchsin mounts; Figs. 6.6,6.8 = wet mounts.).

Figure 6-1. Vegetative hypha directly penetrating a host rhizoid. Scale bar = 10 μm.

Figure 6-2. Appressoria (arrowheads) on a host rhizoid. Scale bar = 10 μm.

Figure 6-3. Protonematal filament being colonized by *A. muscivora*. The fungus has produced an appressorium (white arrowhead) to facilitate host penetration and the host has responded to the attempt by depositing darkly pigmented materials around the intruding hyphae, creating papillae. Scale bar = 10 μm.

Figure 6-4. Rhizoid with a papilla formed in response to a penetration attempt. The penetrating hypha is indicated by an arrowhead. Scale bar = 5 μm.

Figure 6-5. Rhizoid colonized by *A. muscivora*. A branch of the rhizoid has been broken off, exposing intracellular hyphae (arrowheads). Scale bar = 20 μm.

Figure 6-6. Chlorotic leaf cells infected by *A. muscivora*. Scale bar = 20 μm.

Figure 6-7. Leaf in cross section showing cells containing hyphae (arrowheads). Scale bar = 12 μm.

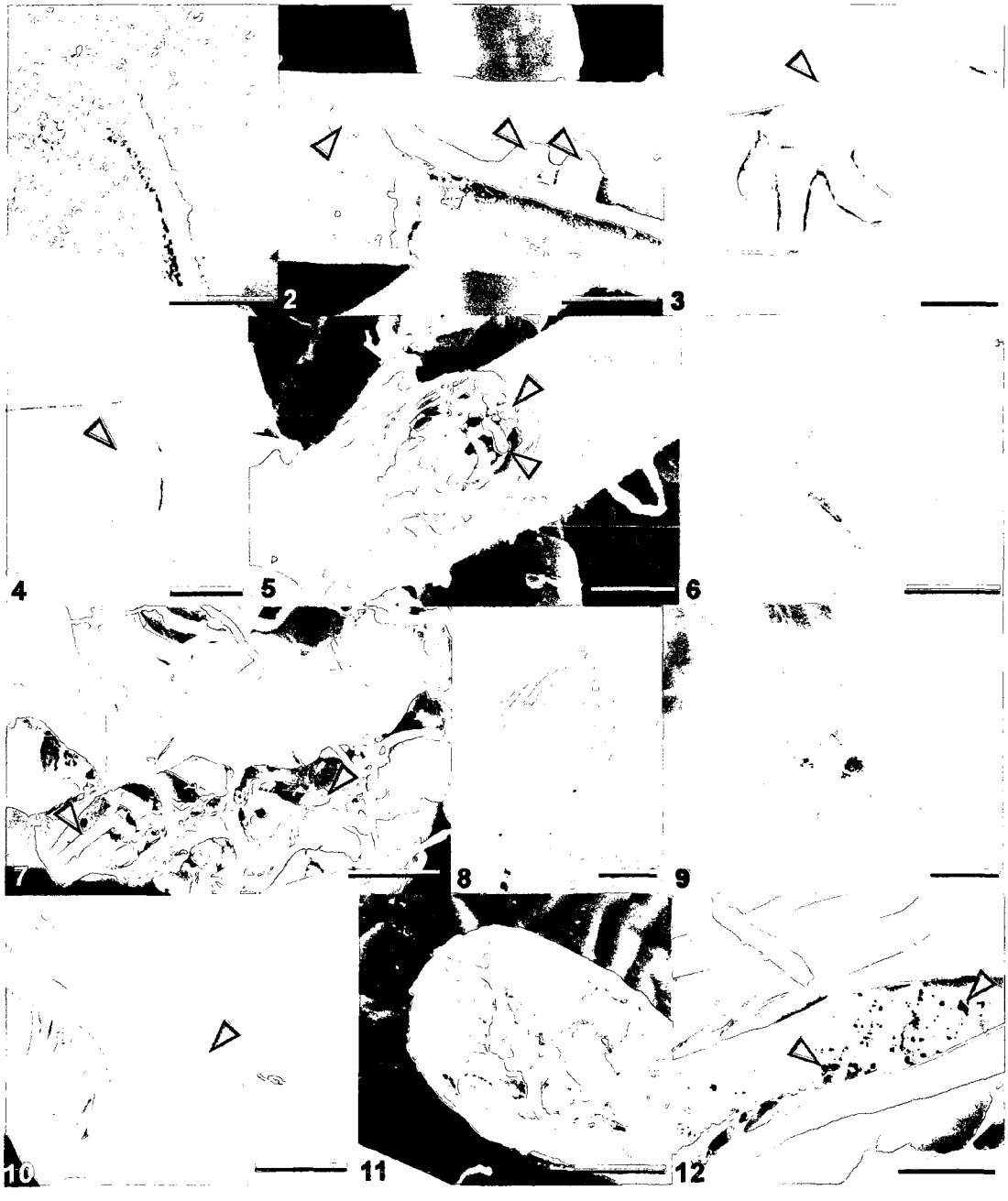
Figure 6-8. Heavily infected *Funaria* leaf that has become chlorotic and mottled due to host response. Scale bar= 400 μm.

Figure 6-9. Mottled, chlorotic gametophyte infected with *A. muscivora*. Scale bar = 1 mm.

Figure 6-10. Hypha (arrowhead) growing between the lamellate layers of the host cell wall. Scale bar = 5 μm. Note: Lamellations of cell wall are visible in Fig. 6.5.

Figure 6-11. Rhizoid tip infected by *A. muscivora* showing generalized wall thinning. Scale bar = 13 μm.

Figure 6-12. *Funaria* leaf cell showing the formation of localized voids (arrowheads) where the cell wall material has been degraded. Scale bar = 10 μm.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Figures 6-13 to 6-22. Development of pycnidia and pseudothecia of *Atradidymella muscivora* on the model host *Funaria hygrometrica*.(Figs. 6-13 to 6-15, 6-21, 6-22 = UAMH 10909; Figs. 6-16 to 6-19 = UAMH 10911; Figs. 6-13, 6-21 =Lactofuchsin mount; Figs. 6-14, 6-19 = wet mount; Figs. 6-15, 6-16 = SEM; Figs. 6-17, 6-18, 6-20, 6-22 = Paraffin section stained with Safranin O- Fast Green FCF)

Figure 6-13. Leaf cell containing a tightly packed hyphal proliferation (arrowhead) of *A. muscivora* Scale bar = 35 μm.

Figure 6-14. Young pycnidium (arrowhead) emerging from a leaf cell. Scale bar = 40 μm.

Figure 6-15. Young pycnidium emerging from a stem cell. Scale bar = 17 μm.

Figure 6-16. Maturing pycnidium on a leaf of *Funaria*. Scale bar = 20 μm.

Figure 6-17. Longitudinal section of a young pycnidium erumpent from a rhizoid. Scale bar = 25 μm.

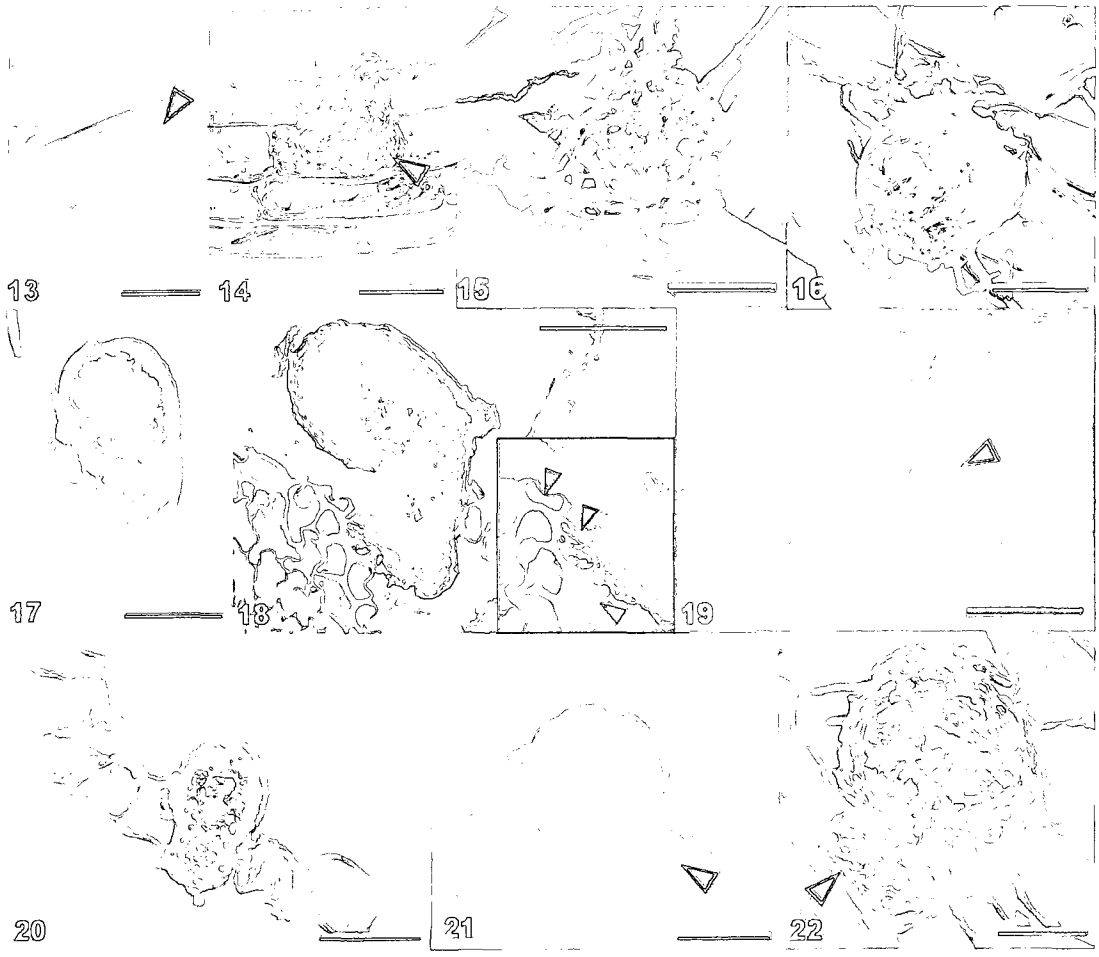
Figure 6-18. Section of *Funaria* stem infected with *A. muscivora*. Scale bar = 85 μm. Inset shows entire pycnidium is erumpent from a single cell (white arrowhead) and adjacent cells are not disrupted (black arrowheads).

Figure 6-19. Mature pycnidium (arrowhead) releasing a cirrhus of conidia into distilled water. Scale bar = 150 μm.

Figure 6-20. Cross section of a developing stroma on a leaf. Scale bar = 30 μm.

Figure 6-21. Young pseudothecium and stroma (arrowhead). Scale bar = 30 μm.

Figure 6-22. Mature pseudothecium showing a jumbled mass of unliberated ascospores within the pseudothecium and a stroma contained within a single host cell (arrowhead). Scale bar = 35 μm.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Figures 6-23 to 6-31. Infection of *Aulacomnium palustre, Hylocomium splendens,* and *Polytrichum juniperinum* by *Atradidymella muscivora.* (Fig. 6-23 = UAMH 10910; Fig. 6-24 = Ap1-Q; Figs. 6-25 to 6-29 = Ap1-S; Figs. 6-30, 6-31 = UAMH 10909; Figs. 6-23, 6-30, 6-31 = SEM; Fig. 6-24 = Lactofuchsin mount; Figs. 6-25, 6-28, 6-29 = Paraffin section stained with Safranin O – Fast Green FCF; Figs. 6-26, 6-27 = Wet mount)

Figure 6-23. Appressorium (arrowhead) on a leaf cell of *Hylocomium*. Scale bar = 6 μm.

Figure 6-24. Rhizoid of *Aul. palustre* that has formed papillae (arrowheads) in response to penetration attempts. Scale bar = 10 μm.

Figure 6-25. Cross section of a stem of *Aul. palustre* showing a pycnidium emerging from a single epidermal cell (arrowhead). Scale bar = 75 μm. Inset shows an enlarged view of the pycnidium emerging from the epidermal cell.

Figure 6-26. Apex of a gemma stalk of *Aul. palustre* with pycnidia (arrowhead) on its gemmae. Scale bar = 650 μm.

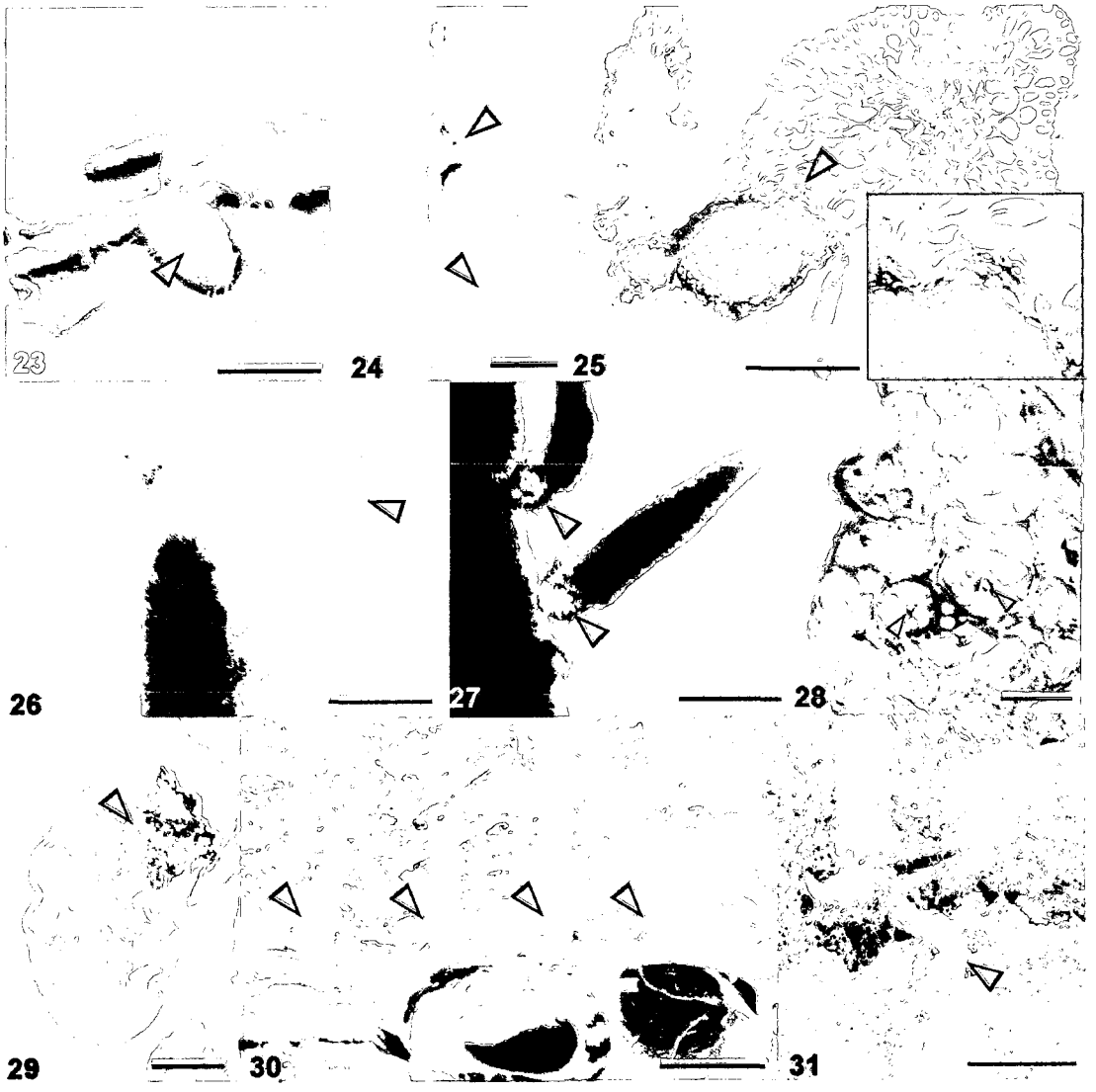
Figure 6-27. Gemma stalk of *Aul. palustre* showing pycnidia (arrowheads) emerging from stem cells and replacing the gemmae. Scale bar = 250 μm.

Figure 6-28. Longitudinal section of heavily infected gemma of *Aul. palustre* with papillae (arrowheads) formed in response to intracellular hyphal growth. Scale bar = 20 μm.

Figure 6-29. Oblique section of gemma of *Aul. palustre* showing a pycnidium emerging from a single cell (arrowhead). Scale bar = 50 μm.

Figure 6-30. Abaxial side of a leaf of *Pol. juniperinum* with hyphae (arrowheads) growing between the lamellate layers of the cell wall. Scale bar = 20 μm.

Figure 6-31. Leaf cell of *Pol. juniperinum* exhibiting generalized wall thinning and localized void formation around an invading hypha (arrowhead). Scale bar = 10 μm.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Chapter 7

Evidence that *Coniochaeta velutina* is a latent saprobe and parasite of mosses.

Introduction

Bryophytes play host to a wide variety of cosmopolitan, pathogenic and saprobic fungi that have also been reported in association with vascular plants, in addition to a number of apparently bryophyte-specific pathogens and parasites (Racovitza 1959, Felix 1988, Kost 1988, Döbbeler 1997, Tosi et al. 2002, Davey and Currah 2006). Although common vascular plant pathogens, including *Phoma* species (Tosi et al. 2002, McRae and Seppelt 1999), *Botrytis cinerea*, *Nodulisporium* species (Thormann et al. 2001), and *Stemphylium botryosum* (Prior 1966), have been reported from mosses, it is not known whether they also infect and cause disease in bryophyte hosts. Fungal disease etiology in bryophytes has not been well characterized and it is not clear if it is homologous to that of vascular plants. However, similarities between bryophilous and vascular plant pathogens in penetration and absorption structures formed during pathogenesis (Döbbeler 1997, Davey et al. Unpublished 2008), and the presence of homologues to vascular plant resistance genes in the model bryophyte *Physcomitrella patens* (Akita and Valkonen 2002, Andersson et al. 2005, de Leon et al. 2007), suggest pathogenesis and host responses may be homologous among the embryophytes.

Coniochaeta velutina is a member of the Coniochaetales (Sordariomycetidae) that is characterized by its small, setose perithecia; darkly pigmented, ovoid ascospores with a single longitudinal germ slit; and the production of a *Lecythophora* anamorph with

hyaline, ovoid conidia and short phialides that are often reduced to adelophialides (a lateral collarette on the vegetative hyphae) (Taylor 1970, Weber 2002). The species has been reported as a saprobe inhabiting a wide variety of plant substrates, soil, and herbivore dung (Taylor 1970, Weber 2002) and a putative endophyte or weak pathogen of a variety of conifers and woody angiosperms (Basham et al. 1969, Taylor 1970, Checa et al. 1988, Hutchison and Reid 1988), but has not been previously described from moss substrates. During a taxonomic survey of fungi inhabiting boreal mosses, an isolate of *Coniochaeta velutina* was obtained, and its interactions with a moss host, *Funaria hygrometrica*, were characterized *in vitro* by light (LM) and scanning electron microscopy (SEM).

Materials and Methods

Gametophytes of *Hylocomium splendens* were collected at Moe's Bog, a wetland 2km southwest of Hoselaw, Alberta, Canada (SW6-60-6 W4). Gametophytes were surface sterilized, fragmented and plated on mineral agar (MIN: 4.4 g/L K_2HPO_4, 1.7 g/L KH_2PO_4, 2.1 g/L NH_4Cl, 0.195 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, 0.05 g/L $FeSO_4$, 0.003 g/L $CaCl_2$, 15 g/L agar) amended with 0.01% oxytetracycline (Sigma, St. Louis, MO, USA) for isolation, as described by Davey and Currah (2007) and a single isolate of *Coniochaeta velutina* (UAMH 10912) was recovered. Disease etiology in *Hylocomium splendens* infected by other bryophilous pathogens is similar in the model host *Funaria hygrometrica* (Davey et al. 2008, unpublished). Because *H. splendens* is slow growing and difficult to establish in axenic culture, *F. hygrometrica* was used as a model system to investigate the interactions between *C. velutina* and bryophytes.

Funaria hygrometrica was cultured *in vitro* from spores using the methods described in Davey and Currah (2007). Gametophytes at the 5 leaf stage were inoculated with *C. velutina* (UAMH 10912) by cutting one or two 5 mm diameter plugs from just behind the leading margin of 15 - 30 day old colonies grown on oatmeal agar (OA: 20 g/L ground oatmeal, 20 g/L agar) and placing them between the tightly packed gametophytes of the moss colony. Inoculated gametophytes, and uninoculated controls were incubated for 12 weeks at 15°C and 75% relative humidity on a 12 hour light, 12 hour dark cycle, and observed by LM weekly. Important stages in the disease etiology were selected for further examination by LM and SEM.

Weekly observations were made by stereomicroscope and by mounting individual leaves or entire gametophytes in water or polyvinyl alcohol with acid fuchsin (0.05g acid fuchsin in 10mL lactic acid and 1mL glycerine mixed with 1.66g polyvinyl alcohol dissolved in 10mL water) and observing by LM. Infected host tissues were fixed in FAA or 2% glutaraldehyde for at least 24 hours, dehydrated in an ethanol series, and embedded in paraffin wax. Sections were stained using Safranin O (Sigma, St. Louis, MO, USA), counterstained with Fast Green FCF (Sigma, St. Louis, MO, USA), and mounted using DPX mountant (Sigma, St. Louis, MO, USA). Light micrographs were taken using an Olympus BX50 microscope with a DP-12 digital camera.

For scanning electron microscopy (SEM), infected gametophytes were fixed in 2% glutaraldehyde (Sigma, St. Louis, MO, USA) overnight, rinsed in distilled water, placed in 2% tannic acid-2% guanidine hydrochloride (Sigma, St. Louis, MO, USA) solution for 4-5 hours, and then postfixed overnight in 2% OsO_4 (Sigma, St. Louis, MO,

USA) at 5°C. Dehydration in an ethanol series and amyl acetate was followed by critical-point drying in a BAL-TEC CPD 030 dryer using carbon dioxide. Dried samples were sputter coated with gold and examined with a Hitachi S-510 scanning electron microscope at 10 or 15 kV.

Enzyme characterization – *Coniochaeta velutina* (UAMH 10912) was tested for amylase, gelatinase, pectinase, cellulase, lipase, and insoluble and soluble polyphenolic oxidase enzyme activities using indicator media, as described by Davey & Currah (2007).

Sequence Comparison – The LSU and SSU regions of the genomic rRNA gene complex were amplified as described in Davey & Currah (2007) using the forward and reverse primer sets NS1 and NS8 (White et al. 1990), and LROR and LR7 (Vilgalys and Hester 1990), respectively. Small subunit rRNA amplicons were sequenced using the primer set NS1, NS2, NS3, NS4, NS6 (White et al. 1990), NS13, and NS 151, and large subunit rRNA amplicons were sequenced using the primer set LROR, LR7, LR3R, LR5, and LR16 (Vilgalys and Hester 1990). Pairwise comparison of the LSU and SSU regions to published sequence data retrieved from GenBank for *C. velutina* isolated from vascular plants was conducted using bl2seq (Tatusova and Madden 1999). The ITS region of the genomic rRNA was not investigated, because there are no ITS sequences of *C. velutina* available from GenBank, nor is the type specimen available for sequencing and comparison, and previous phylogenetic studies of the Coniochaetales have used the LSU regions of the genomic rRNA (Weber et al. 2002).

Results

Within 10 days of inoculation, sparse white mycelium was evident on the surface of rhizoids, although there were no macroscopic signs or symptoms of disease. This mycelium produced short phialides, adelophialides, and ovoid to elliptic conidia 1-1.5 x 2.5-5 μm 7-15 days post inoculation (Fig. 7-1) and continued to produce conidia for the entire duration the host was observed. Within 7-10 days of inoculation, vegetative hyphae were observed directly penetrating the cell walls of rhizoids (Fig. 7-2). Small, sunken, degraded areas of cell wall were often present around penetrating hyphae (Fig. 7-2) and occasionally extended in a halo around actively growing hyphae. Within rhizoids, sites of fungal ingress had successive layers of darkly pigmented material deposited around the intruding hypha, ultimately creating unbranched, papilla-like structures (Fig. 7-3). Intracellular hyphae were not observed within cells containing these structures. Twenty days post-inoculation, hyaline hyphae were observed growing intracellularly in rhizoids and protonemata lacking papilla-like structures (Figs. 7-4, 7-5), and 25-30 days post-inoculation, these intracellular hyphae produced phialides and conidia identical to those produced at the surface of the gametophytes. Infected rhizoids became packed full of conidia (Fig.7-6). Rhizoids cells that did not contain intracellular hyphae and lacked intact protoplasts were observed in both inoculated mosses and controls. Intracellular hyphae were not observed within leaves or stems (Fig. 7-7).

Gametophytes either remained macroscopically asymptomatic or exhibited mild chlorosis 30-60 days post-inoculation. Perithecia formed on the surface of the rhizoids 30-40 d after inoculation (Fig. 7-8) and were not observed on stems or leaves. Perithecia

were initiated from an interwoven mat of swollen hyphae that was tightly appressed to the rhizoid walls, sometimes completely encircling the rhizoid (Fig. 7-9). The hyphal mat first became dematiaceous (Fig. 7-10), then pseudoparenchymatous, as it developed into a perithecium initial (Fig. 7-11). Intracellular hyphae did not appear to contribute to the perithecium initials, although subsequent growth and expansion of the perithecium often caused the rhizoid to appear embedded in the peridium (Fig. 7-12). Perithecia matured 10-20 d after initiation, and the 8-spored unitunicate asci developed and dehisced asynchronously (Fig. 7-13). Ascospores were forcibly discharged and mature perithecia remained attached to the rhizoids, even after ascospore discharge had occurred (Fig. 7-14). Liberated ascospores often adhered in clumps to the surface of the leaves and rhizoids, causing them to appear speckled.

Enzyme production - The fungus tested positively for cellulase and soluble polyphenolic oxidase activities. Tests for amylase, pectinase, gelatinase, lipase, and insoluble polyphenolic oxidase activities were negative.

Phylogenetic analysis – The isolate of *C. velutina* from *Hylocomium splendens* shared 100% sequence similarity across the 18S SSU region, and 99% similarity across the 28S LSU region to isolates of *C. velutina* from *Picea abies* (GenBank #: EU999180, EU999181, AJ496244, AF353594).

Discussion

Coniochaeta velutina is a weak pathogen that causes wood-staining of tree species (Basham et al. 1969, Hutchison and Reid 1988) and has also been reported as a saprobe of vascular plant material and herbivore dung (Checa et al. 1988, Weber 2002). Based on

its interactions with *Funaria hygrometrica, C. velutina* can be considered a weak, opportunistic parasite or latent saprobe of mosses. Infected gametophytes did not die, infection was limited to the rhizoids, and symptoms of infection (i.e. mild chlorosis) were manifest only after long incubation periods (30-60 d), if at all, suggesting that the fungus, while capable of penetrating and persisting within its host, is a weak, opportunistic parasite that is neither lethal nor extremely virulent. Isolates of *C. velutina* associated with vascular plants are known to produce lignocellulose degrading enzymes (Lopez et al. 2007) and our isolate also produces both cellulase and polyphenolic oxidase enzymes. This, in conjunction with the localized cell wall degradation observed in live *F. hygrometrica* indicates the fungus may also be classed as a latent saprobe that has little impact on the function of its host, but would be capable of degrading cell wall materials upon host death.

The direct penetration of host cells by vegetative hyphae observed in *C. velutina* infecting *Funaria* is consistent with mechanisms of host penetration that have been described in fungal pathogenesis of vascular plants (Agrios 1997, Cole and Hoch 1991). Attempts to penetrate live cells were evidenced by the presence of papillae formed by the host's defense response; however, they do not appear to be successful, as intracellular hyphae were not observed traversing host papillae or in cells that had intact protoplasts. Given the weak pathogenicity and saprobic nature of *C. velutina* on angiosperm tree hosts, and the reported similarities between bryophyte and vascular plant defense responses (Davey et al. 2008 unpublished), the papilla host response is likely sufficient to prevent infection of live bryophyte cells, accounting for the absence of intracellular

hyphae in stems, leaves, and rhizoids with intact protoplasts. As such, *C. velutina* is most likely able to successfully penetrate only non-living cells, and colonizes bryophyte hosts because they are a heterogenous composition of living, moribund and dead cells. The lower stems of mosses are frequently senescent (Bates 1998), and in particular, *Hylocomium splendens*, the host from which *C. velutina* was isolated, forms thick carpets in which only the uppermost part of the gametophyte is photosynthetically active, and the lower branches, stems, leaves, and rhizoids are moribund or dead. The presence of rhizoids lacking intact protoplasts in both infected and control gametophytes suggests a subset of the rhizoids of *F. hygrometrica* grown *in vitro* are senescent, moribund or dead, serving primarily storage (Hakala and Sewon 1992), anchoring, and/or capillary water conduction functions (Malcolm and Malcolm 2006). The abundance of senescent tissues on both its native host and the model host *F. hygrometrica* provide ample substrate for colonization by *C. velutina* and allow it to circumvent the host's defence responses.

In response to fungal ingress, *Funaria* deposits layers of darkly pigmented material around the intruding hypha, ultimately forming a structure morphologically indistinguishable from the callose- and polyphenolic-rich papillae formed by vascular plants in response to penetration attempts by fungi (Cole and Hoch 1991, Agrios 1997). This host response appears to be a common, generalized resistance mechanism to fungal infection, and has previously been reported in *F. hygrometrica* (Martinez-Abaigar et al. 2005, Davey et al. Unpublished 2008) and a taxonomically diverse suite of other mosses (Racovitza 1959, Hassel and Kost 1998), as well as in hepatophytes (Racovitza 1959, Read et al. 2000). The apparent universality of this host response is consistent with

genetic studies that suggest homology in pathogenesis and disease resistance among bryophytes and vascular plants (Akita and Valkonen 2002, Andersson et al. 2005, de Leon et al. 2007). Although *C. velutina* has been reported as an opportunistic pathogen of tree species that infects its hosts through wounds or cankers (Basham et al. 1969, Hutchison and Reid 1988), detailed anatomical studies have not been conducted to determine if it is also able to directly penetrate its vascular plant hosts and whether a papilla formation host-response similar to that of *Funaria* occurs, forcing the fungus to rely on opportunistic colonization of wounds and cankers to infect its hosts. Penetration of vascular plant hosts by *C. velutina* could also potentially be precluded by differences in cell wall composition and biochemistry (Wilson et al. 1989) between vascular plant and bryophyte hosts, or by unfavourable growing conditions on the periderm of tree hosts that do not provide sufficient nutrients to support growth of *Coniochaeta* species (Wang et al. 1997). Detailed studies of the host-fungus interface and disease etiology of *C. velutina* infecting tree species are needed to determine if pathogenesis and host responses are homologous between the vascular plant and bryophyte hosts infected with this fungus.

The ovoid conidia, short phialides, and adelophialides that were produced both on and within host gametophytes are consistent with descriptions of the *Lecythophora* anamorph of *C. velutina*. The anamorph sporulated within its host's rhizoids, a phenomenon not previously described among bryophilous hyphomycetes. Anamorphic fungi inhabiting mosses more typically produce an intracellular mycelium that functions in nutrient acquisition, and then sporulate at the surface of the plant where their conidia are easily dispersed (Racovitza 1959). It is not clear for the *Lecythophora* anamorph of

C. velutina whether the conidia are dispersed only when rhizoids are broken, damaged, or degraded, or if the expansion of the conidial mass causes the rhizoids to rupture, allowing for spore dispersal, much in the same way pressure created by developing conidia during acervulus formation in coelomycetes eventually ruptures the host epidermis, allowing spore dispersal (Kendrick 1992).

Coniochaeta velutina produces superficial ascomata on its vascular plant hosts, and is typically found at cankers or wound sites, or fruiting superficially on twigs and rotting wood (Basham et al. 1969, Taylor 1970, Hutchison and Reid 1988, Weber 2002). On *F. hygrometrica*, *C. velutina* produces ascomata that are superficial, in that intracellular hyphae do not contribute to their formation. However, in contrast to ascoma development on vascular plant hosts, the original bryophyte rhizoid cell associated with a developing ascoma frequently becomes embedded in the mature ascoma's peridium. The incorporation of host tissues into the mature peridium does not appear to be reflective of host-dependent differences in peridium development and is likely due to the small size and uniseriate nature of bryophyte rhizoids compared to large, multi-cellular vascular plant tissues.

The high degree of sequence similarity between isolates of *C. velutina* from mosses and vascular plants may be reflective of the existence of strains capable of infecting both bryophyte and vascular plant hosts. As a wood-staining pathogen, *Coniochaeta velutina* has not been observed to actively penetrate its vascular plant hosts, and instead appears to opportunistically exploit pre-existing wounds in order to gain entrance into the plant (Basham et al. 1969, Hutchison and Reid 1988). Understory forest

mosses like *Hylocomium splendens* that are a mosaic of living, moribund and dead cells may represent a biological reservoir of inoculum for *C. velutina*, where the fungus can continue to persist and reproduce in the absence of susceptible host trees. Further research into bryophytes as alternate hosts for vascular plant pathogens may elucidate another role of bryophytes in their ecosystems as inoculum reservoirs.

Figures 7-1 to 7-7. Host penetration and anamorph production of *Coniochaeta velutina* infecting *Funaria hygrometrica.*

Figure 7-1. SEM of surface of host rhizoid with superficial mycelium that has produced the *Lecythophora* anamorph of *C. velutina.* Arrowhead indicates a single conidium. Scale bar = 15 μm.

Figure 7-2. Vegetative hypha penetrating the surface of a host rhizoid. Note area of cell wall degradation surrounding the penetration site. Scale bar = 5 μm.

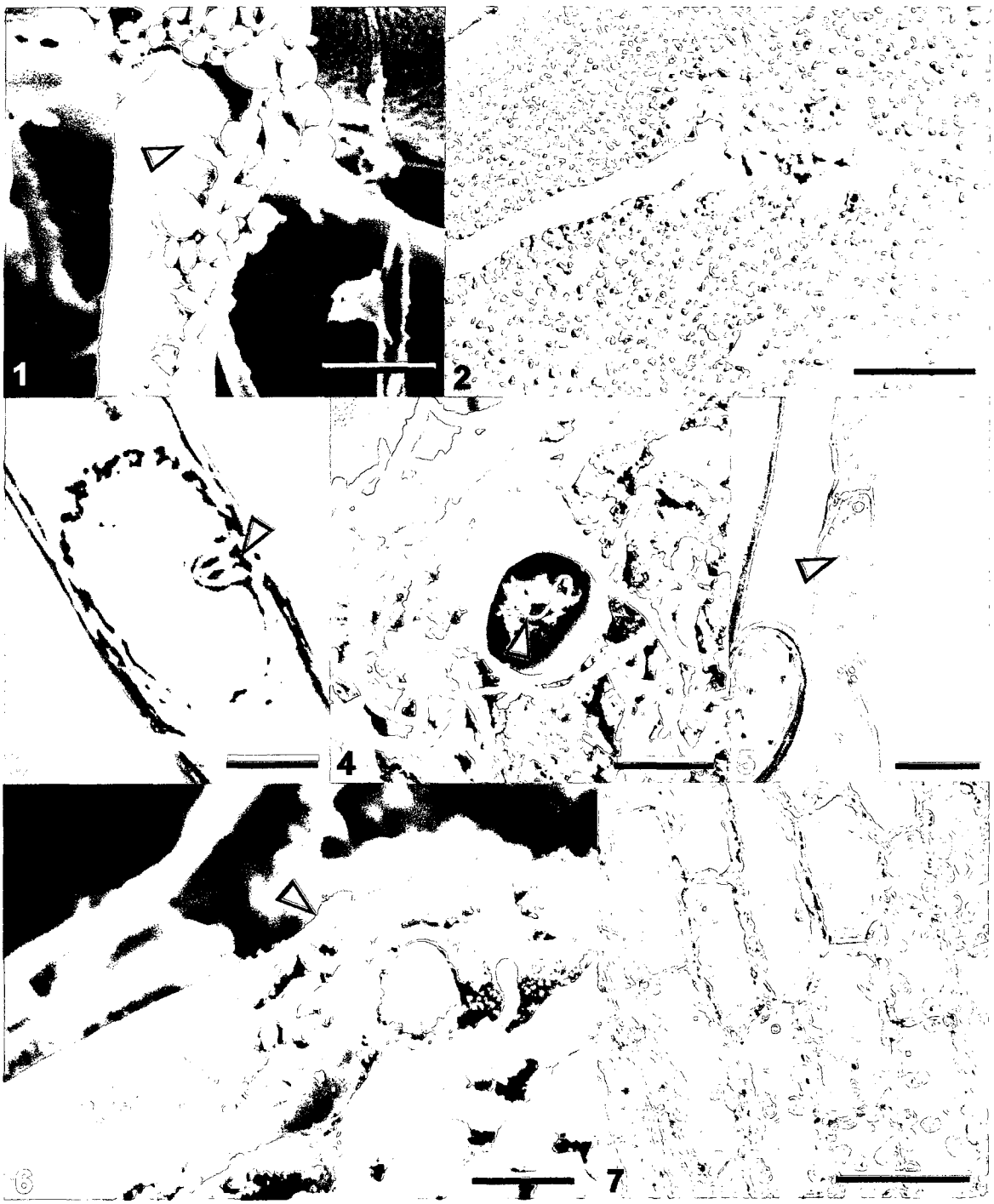
Figure 7-3. Rhizoid exhibiting a papilla surrounding an invading hypha (arrowhead). Scale bar = 17 μm.

Figure 7-4. Host rhizoid surrounded by mycelium and containing intracellular hyphae (arrowhead). Scale bar = 20 μm.

Figure 7-5. Protonematal filament containing hyphae (arrowhead) that are growing intracellularly. Scale bar = 25 μm.

Figure 7-6. Rhizoid containing conidia (arrowhead) of the *Lecythophora* anamorph of *C. velutina.* Scale bar = 6 μm.

Figure 7-7. Leaf cells of infected gametophyte. Note the absence of intracellular hyphae. Scale bar = 35 μm.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Figures 7-8 to 7-14. Teleomorph production in *Coniochaeta velutina* infecting *Funaria hygrometrica.*

Figure 7-8. Infected gametophytes with perithecia (arrowheads) on the surface of rhizoids. Scale bar = 6 mm.

Figure 7-9. Host rhizoid with dense mat of hyaline hyphae that will ultimately form perithecium. Scale bar = 50 μm.

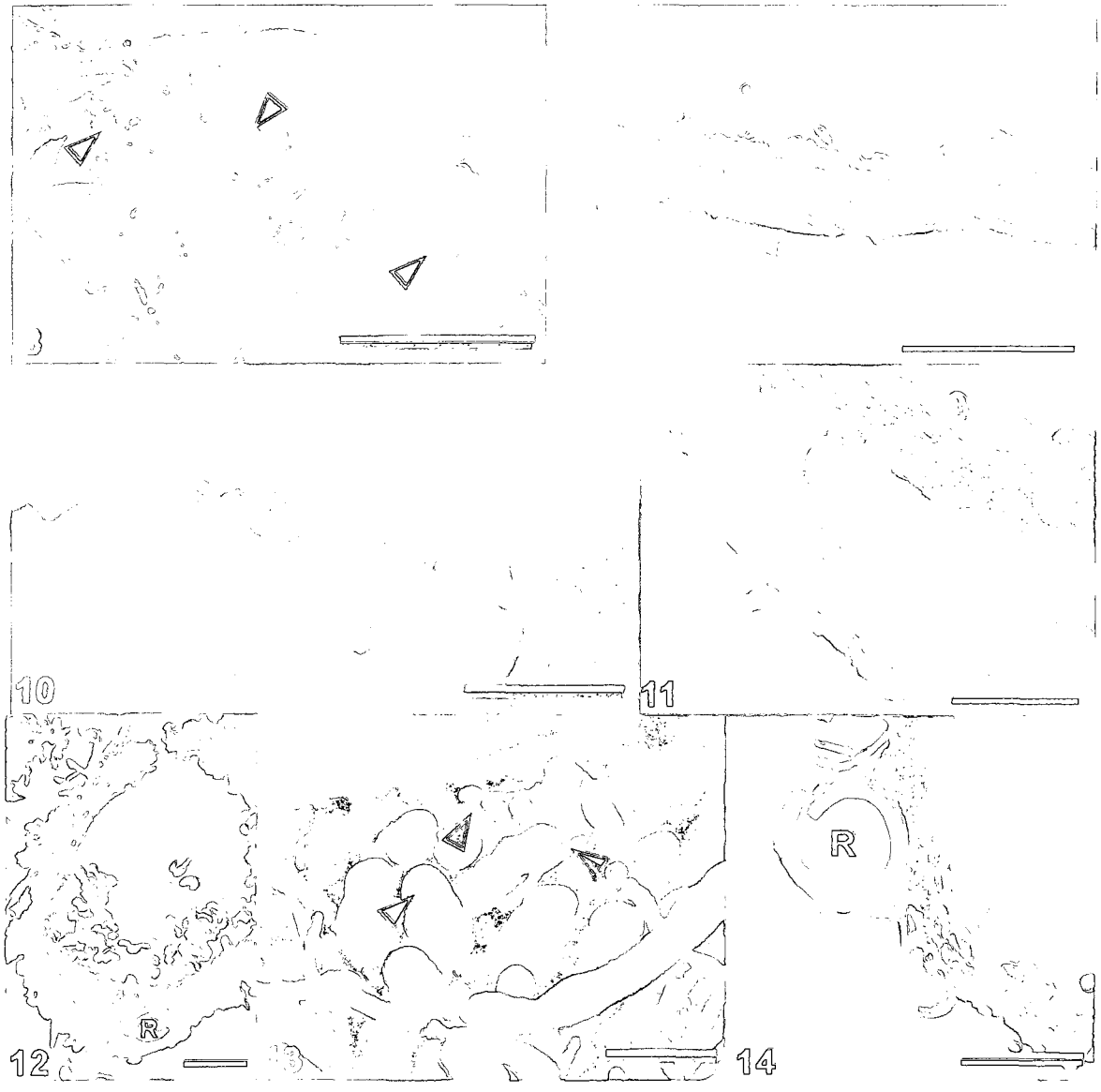
Figure 7-10. Hyphal mat has become melanized and is beginning to become pseudoparenchymatous. Scale bar = 25 μm.

Figure 7-11. Young perithecium with pseudoparenchymatous tissue that completely encircles host rhizoid. Scale bar = 30 μm.

Figure 7-12. Mature perithecium with host rhizoid (R) embedded in peridium wall. Scale bar = 25 μm.

Figure 7-13. Perithecium centrum showing mature asci (white arrowhead), dehisced asci (black arrowhead), and paraphyses (bicolour arrowhead). Scale bar = 7 μm.

Figure 7-14. Mature perithecium after ascospore dispersal that is still attached to host rhizoid (R). Scale bar = 25 μm.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Chapter 8

Conclusions and future research directions

Historically, the study of bryophilous fungi has been sporadic and often lacking in direction. This lack of direction can be attributed to the past misconception that associations between moss and fungi are uncommon and rare and arose due to the study of moss specimens in herbaria that are collected with a bias for healthy plants, an abundance of minute, parasitic fungi causing few macroscopic symptoms on their hosts, and a lack of cooperation between bryologists and mycologists to identify and characterize the respective bionts of these associations. The scattered reports and descriptions of bryophilous fungi have been generally summarized in reviews of taxonomic diversity of ascomycetes and basidiomycetes found in association with mosses. However, reports of the ecological functions of these fungi have remained scattered, providing the impetus for the first research objective of this thesis: to make a comprehensive review of the literature pertaining to the ecological functions of bryophilous fungi in order to delimit gaps in our current knowledge of the group.

In the course of the literature review described in Chapter 2, two main deficiencies in the breadth of our current knowledge of bryophilous fungi were identified. First, cryptic, or inconspicuous fungi associated with mosses have remained almost entirely uninvestigated. While taxonomic diversity among bryophilous fungi forming sexual fruiting structures on mosses has been circumscribed in relatively good detail from morphological studies, only a handful of studies have used techniques that allow the detection of those cryptic associates of mosses that do not form ostentatious fruiting

structures or are sterile. Second, while many fungal taxa have been described as pathogens, parasites, or saprobes of bryophytes, very few studies have investigated the host-fungus interfaces and etiology of these associations. The identification of these deficiencies provided direction and focus for the remaining two research objectives: determining the taxonomic diversity of bryophilous fungi and characterizing their ecological functions.

Given that the morphological identification of sporulating isolates yielded 27 new records of previously described fungi in association with mosses, as well as the description of *Cladophialophora minutissima* sp. nov (Chapter 4) and *Atradidymella muscivora* gen. et sp. nov (Chapter 5) from among these fungal isolates, culture-based techniques represent an invaluable method for detecting species diversity among bryophilous fungi.

Sterile isolates from bryophytes have not been previously characterized, and the use of culturing techniques in combination with molecular techniques as described in Chapter 3 represents a new approach to detecting biodiversity among bryophilous fungi. This approach identified diversity among bryophilous fungi that has remained mostly undetected in the morphological-based approaches that have been employed to date. Given that only 31 sterile or morphologically ambiguous isolates were characterized in Chapter 3, it is expected that more thorough studies using this culture-based molecular approach to identify greater numbers of isolates from additional hosts would reveal additional biodiversity among bryophilous fungi. Furthermore, the success of this small, molecular-based study in detecting previously unknown diversity among fungi associated

with mosses suggests that other culture-free molecular techniques, such as pyrosequencing and environmental PCR, could be used to effectively and thoroughly characterize the cryptic, endophytic fungal communities of bryophytes, and help to circumscribe the taxonomic diversity of bryophilous fungi.

The third research objective of this thesis was to characterize in detail the ecological function of selected bryophilous fungi. In Chapter 4, *Cladophialophora minutissima* was characterized as a latent, endophytic saprobe based on observations of infected hosts and extracellular enzyme profiling that indicated the fungus is capable of degrading both the cellulosic and polyphenolic components of moss cell walls. While extracellular enzyme profiling and general observations by light microscopy provide good evidence for *C. minutissima* as a latent saprobe of mosses, observations of cell wall degradation by electron microscopy and characterization of the infection etiology host senescence are needed to make a truly cogent argument for this ecological role.

In Chapters 6 and 7, the interactions between *Atradidymella muscivora* and the opportunistic tree pathogen *Coniochaeta velutina* and their respective bryophyte hosts were characterized in detail using extracellular enzyme profiling and observations by light and scanning electron microscopy. These studies represent the first detailed characterizations of infection etiology of ascomycetous pathogens *in vitro* using axenically grown moss hosts. This method proved to be an effective way to observe the complete infection etiology from penetration and infection, to reproduction and dispersal, and demonstrates great potential for the *in vitro* study of fungus-bryophyte interactions.

Atradidymella muscivora was determined to be a pathogen of bryophytes and exhibited adaptive features that enable its colonization of bryophyte hosts, including a reduced stroma for avoidance of host defence responses, and initiation of reproductive structures in nutrient-rich microniches. By comparison, *Coniochaeta velutina*, a known opportunistic pathogen of vascular plants, was found to be a weak parasite and latent saprobe of mosses. The fungus appeared only able to infect senescent and moribund bryophyte tissues and did not appear to negatively impact its host, suggesting a new ecological function for bryophytes as inoculum reservoirs for vascular plant pathogens. In both *A. muscivora* and *C. velutina*, host responses to hyphal penetration attempts resembled those that occur during fungal pathogenesis of vascular plants, providing impetus for ultrastructural comparisons of reponses to fungal attack in vascular and non-vascular plants, as well as further investigations into the evolution of mechanisms of resistance in plants.

The research undertaken in Chapters 3-7 of this thesis only begin to address the deficiencies in our knowledge of bryophilous fungi that were identified in the literature review outlined in Chapter 2. However, given the results of this thesis, research on bryophilous fungi should continue in the following broad areas:

1) Taxonomic diversity of fungi associated with bryophytes is not yet fully understood. Continued use of culture-based approaches and employment of culture-free molecular techniques will help to circumscribe this diverse group of fungi.

2) The ecological functions of bryophilous fungi are still not well known. The development of a model system for studying interactions of fungi and bryophytes provides the opportunity to characterize in detail the host-fungus interface and ecological function of more bryophilous fungi. In particular, the ecological function of sterile and cryptic associates of mosses is virtually unknown, and should be explored and compared to the function of cryptic endophytes in vascular plants.
3) The existence of model systems for both functional and genetic studies of mosses provides an excellent platform for study of the evolution of both pathogenesis in fungi and host defense responses in plants.

Further investigation of these areas will undoubtedly reveal further taxonomic and functional diversity among bryophilous fungi, and provide insight into the evolution of plant fungus interactions.

Appendix 1 - Glossary

The following is a glossary of terms used throughout the body of this thesis.

Acervulus – a disc-shaped asexual reproductive structure that is initiated sub-epidermally and eventually ruptures the epidermis of its vascular plant hosts.

Adelophialide – a reduced phialide, often no more than a colarette borne on a vegetative hypha.

Anamorph – the imperfect or asexual stage of a fungus.

Ascoma – the sexual fruiting body produced by Ascomycetes that bears or contains asci and ascospores.

Ascomycete – a member of the Ascomycota.

Ascomycota – a phylum in the kingdom Mycota in which sexual spores (ascospores) are produced within asci.

Ascospore – spores that are formed within asci as a product of meiosis (sexual reproduction). Occur in the Ascomycota.

Ascus – a sac-like cell that is the site of meiosis and contains ascospores (the products of meiosis).

Basidiomycete – a member of the Basidiomycota.

Basidiomycota – phylum in the kingdom Mycota in which sexual spores (basidiospores) are produced exogenously from a basidium.

Bryophilous fungus – a fungus found in close association with a bryophyte host that is not part of the casual phylloplane community.

Bryophyte – member of the division Bryophyta in the kingdom Plantae. Characterized by their lack of vascular tissue, sporophyte that is dependent on the gametophyte generation, and motile male gametes.

Catenulate – occurring in chains.

Coelomycetes – a class of anamorphic fungi that produce conidia within a pycnidium or acervulus.

Conidium – a propagule that is the asexual product of mitosis. Often formed by specialized conidiogenous cells (i.e. adelophialide, annelide, phialide) that are borne on a conidiophore.

Embryophytes – land plants, including both vascular and non-vascular plants.

Endophyte – fungus that grows asymptomatically within living plant tissues.

Gametophyte – haploid stage in the bryophyte life cycle that ultimately bears the gamete-forming organs, and gametes.

Gemma – a multicellular asexual propagule formed by bryophytes.

Haustorium – a specialized, often intracellular, absorptive structure formed by parasitic fungi.

Host – an organism that provides nutrition to another living organism that may be beneficial, detrimental, or neutral in its effects on the host.

Lamellae – thin plate like flaps of tissue on the adaxial side of the costa (midrib) of leaves of members of the Polytrichaceae.

Lamina – the blade of a bryophyte leaf.

Mycelium – collective term for the vegetative hypha forming the thallus of a fungus.

Mycoparasite – a fungus that derives its nutrition from the break-down of other fungi.

Non-vascular plant – members of the divisions Anthocerophyta, Bryophyta, and Hepatophyta in the kingdom Plantae.

Papilla – defense mechanism against fungal infection in vascular plants. Pappillae frequently form around penetrating hyphae and consist of a protuberance of the cell wall into the cell being attacked by the fungus.

Parasite – an organism that derives its nourishment from another living organism (host).

Pathogen – an organism causing disease.

Perithecium – a globular or flask-shaped ascoma with an ostiole through which ascospores are expelled at maturity.

Phialide – a blastic conidiogenous cell producing conidia in basipetal succession from an open terminus.

Phylloplane – the external leaf and stem surfaces of a plant.

Protonemata – typically filamentous juvenile cells produced by germinating bryophyte spores prior to the formation of gametophytes. Protonemata containing chloroplasts are termed chloronemata, while those with fewer chloroplasts are termed caulonemata.

Pseudothecium – an ascoma superficially resembling a perithecium, but arising from the lytic formation of locules within a stroma, followed by the production of asci within these locules.

Rhizoid – filamentous, often pigmented cells on the stems of bryophytes that serve both anchoring and capillary water conduction functions.

Saprobe – an organism that derives its nutrition from the degradation of dead organisms or non-living materials derived from living organisms.

Seta – in fungi, bristle-like appendages often found on ascomata. In bryophytes, the stalk of a sporophyte.

Sporophyte – diploid stage in the bryophyte life cycle that ultimately gives rise to meiotically produced spores. The sporophyte is attached to, and dependent on the gametophyte generation in bryophytes.

Stroma – a compact mass of fungal tissue in which or from which reproductive structures are often formed.

Teleomorph – the perfect, or sexual stage of a fungus.

Vascular plant - a member of the tracheophyte or spermatophyte lineages of the kingdom Plantae.