

## ENDOPHYTIC *XYLARIA* (XYLARIACEAE) AMONG LIVERWORTS AND ANGIOSPERMS: PHYLOGENETICS, DISTRIBUTION, AND SYMBIOSIS<sup>1</sup>

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Nuclear ribosomal 18S and internal transcribed spacer (ITS) sequence data were used to identify endophytic fungi cultured from six species of liverworts collected in Jamaica and North Carolina. Comparisons with other published fungal sequences and phylogenetic analyses yielded the following conclusions: (1) the endophytes belong to the ascomycete families Xylariaceae, Hypocreaceae, and Ophiostomataceae, and (2) liverwort endophytes in the genus *Xylaria* are closely related to each other and to endophytes isolated from angiosperms in China, Puerto Rico, and Europe. Liverwort endophytes are expected to be foragers or endophytic specialists, although little is known about the role of these fungi in symbioses. Features that may indicate a mutualistic role for these endophytes are discussed.

**Key words:** endophytes; Jamaica; liverworts; North Carolina, USA; phylogeny; *Xylaria*; Xylariaceae.

Endophytic fungi that live inside healthy plant tissue without apparent damage to the host are found in all lineages of land plants (Petrini and Petrini, 1985). New species are continually being described from cultural and molecular studies of plant tissue, and endophyte biology is a burgeoning field in mycology. (A Biological Abstracts search for “endophyt\*” in the title retrieved 923 papers from 1990 through January 2003.) These studies indicate that the breadth of endophyte diversity and ecology is just beginning to be discovered (Arnold et al., 2000).

Many groups of fungi exist as endophytes, though most are ascomycetes. Well-known examples are Clavicipitaceae (e.g., *Epichloe*) species that inhabit grasses (Poaceae). Endophytic associations with *Epichloe* have been shown to be mutualistic: the plant receives protection from herbivory through fungal toxins, and the fungus receives host tissue as a nutritive source, along with seed-mediated dispersal of mycelia (reviewed in Clay, 1988). However, the ecology and distribution of most groups of endophytic fungi remain poorly known.

Endophytic Xylariaceae have been documented in conifers, monocots, dicots, ferns, and lycopsids (Brunner and Petrini, 1992). One hypothesis for the role of Xylariaceae endophytes holds that the fungus is a quiescent colonizer and will later decompose cellulose and lignin when the plant begins to senesce (Petrini et al., 1995; Whalley, 1996). However, growing evidence suggests that some xylariaceous fungi may exist solely as endophytes (Rogers, 2000; J. D. Rogers, Washington State University, personal communication). No obvious benefit to living host plants has been documented for Xylariaceae.

Liverworts are nonvascular, spore-bearing plants, or “bryophytes.” Though these plants have long been known to form associations with fungi (see Boullard [1988] and Read et al. [2000] for review), few liverwort endophytes have been iden-

tified with certainty. Duckett and Read (1995) grew ascomycetes from 11 British liverworts and through cross-inoculation experiments with angiosperms concluded that the fungi were likely *Hymenoscyphus ericae* (D. J. Read) Korf and Kernan (Leotiaceae), the ascomycete that forms mycorrhizae with the flowering plant family Ericaceae. This species was also identified from an Antarctic liverwort [*Cephaloziella exilifora* (Taylor) Stephani (Cephaloziellaceae)] based on DNA sequences from the nuclear ribosomal internal transcribed spacer (ITS) (Chambers et al., 1999). It is unclear whether Xylariacean endophytes previously isolated from “bryophytes,” as listed in Petrini and Petrini (1985), included any liverworts. Endophytes of some liverwort species are restricted to the rhizoids, while those of other liverwort species can be detected growing within the thallus. Most rhizoid-associated endophytes are thought to be ascomycetes, while those within thalli are thought to be basidiomycetes or Glomalean fungi (Boullard, 1988). The resemblance of these associations to vascular plant mycorrhizae have led some to label them as mutualistic, though the nature of the symbiosis remains poorly understood (Read et al., 2000).

The goal of this study was to characterize the endophytic communities of six common liverworts collected in Jamaica and North Carolina, USA. The study consisted of three parts: (1) morphological observations of the fungal infection, (2) identification of the endophytes based on nrDNA similarity and phylogeny, and (3) ecological comparisons of the endophytes with related fungal species.

### MATERIALS AND METHODS

**Liverwort collections**—Twenty specimens of *Bazzania* (Lepidoziaceae) were collected from five localities in the Blue Mountains of Jamaica. Due to the problematic classification of Neotropical *Bazzania*, collections could not be identified to species. Three specimens of *Calypogeia mulleriana* (Schiffn.) K. Muller (Calypogeiaceae) were collected from one locality in the piedmont of North Carolina. Three specimens of *Odontoschisma prostratum* (Sw.) Trev. (Cephaloziaceae) were collected from two localities in the North Carolina piedmont. One specimen each of *Metzgeria furcata* (L.) Dum. (Metzgeriaceae), *Plagiochila virginica* Evans (Plagiochilaceae), and *Trichocolea tomentella* (Ehrh.) Dum. (Trichocoleaceae) were collected from one locality in

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the mountains of North Carolina. Locality details for vouchers, deposited in DUKE, accompany the online version of this article.

**Cultures**—Cultures were established following a modified version of the procedure from Arnold et al. (2000), in which contamination from surface fungi was minimized by submersion of the plant tissue in a 5% bleach solution for 2 min, followed by submersion in 70% ethanol for 2 min. Liverwort fragments were plated on sterile potato dextrose agar or malt extract agar using aseptic technique. Pure living cultures of all fungi are vouchered at DUKE and will be submitted to a public culture collection pending morphological identification.

**Molecular methods**—Total genomic DNA was extracted from cultured fungi using the method of Doyle and Doyle (1987). The ITS 1, 5.8s, and ITS 2 regions of rDNA were amplified using the primers ITS 1 and ITS 4 (White et al., 1990), and the 18S region was amplified using the primers NS 1 and NS 8 (White et al., 1990). Polymerase chain reactions (PCR) were performed using a Perkin Elmer 480 (Perkin Elmer, Norwalk, Connecticut, USA) with 35 cycles of 94° for 1 min, 50° for 30 s, and 72° for 1 min, with an additional 7-min extension at 72° after cycling. The PCR amplicons were purified using Qiaquick spin-columns (Qiagen, Valencia, California, USA) according to manufacturer protocols.

Sequencing PCR utilized Big Dyes v.2.0 (Applied Biosystems, Foster City, California, USA) and an ABI Prism 3700 (Applied Biosystems). Additional internal 18S primers NS 1.5, NS 2, NS 4 (White et al., 1990), and BMB-BR (Lane et al., 1985) were used to improve sequencing results. All sequences have been submitted to GenBank (see Supplemental Data accompanying the online version of this article).

**Analyses**—Preliminary identifications of fungal ITS sequences were obtained using the GenBank BLAST (Altschul et al., 1997) sequence similarity search with all filters removed. The closest matches were used to identify the major group of fungi to which each sequence belonged and to guide GenBank sampling for 18S phylogenetic analyses.

Alignments of ITS and 18S sequences and GenBank accessions were performed manually using Se-A1 version 1 (A. Rambaut, University of Oxford, Oxford, UK). Regions that could not be unambiguously aligned were excluded from further analysis. The alignments are available upon request from the authors.

Aligned ITS sequences were analyzed using equally weighted parsimony implemented in PAUP 4.0b10 (Swofford, 2002). A branch and bound search was conducted, with gaps scored as missing data. Trees were mid-point rooted.

Aligned 18S sequences were analyzed using equally weighted parsimony and maximum likelihood using PAUP. Heuristic parsimony searches were conducted using 100 random addition replicates with MulTrees and steepest descent in effect. Gaps were scored as missing data. Parsimony bootstrap support values were calculated using 100 full heuristic searches with 10 random additions per replicate (Felsenstein, 1985). The maximum likelihood substitution model for 18S was determined by calculating the likelihood for 56 models and comparing them using likelihood ratio tests, implemented in Modeltest 3.06 (Posada and Crandall, 1998). The best model was Tamura-Nei with equal base frequencies and among-site rate heterogeneity specified by a gamma shape parameter. The likelihood searches were conducted using 100 random addition replicates. Bayesian analyses were conducted on the aligned data set using MrBayes 2.01 (Huelsenbeck and Ronquist, 2001) using a model of equal base frequencies with six substitution types and a gamma shape parameter. Four simultaneous Markov chain Monte Carlo searches were run for 1 000 000 generations and trees were sampled every 100 generations. Plots of the likelihoods from each sample were made to determine the number of generations until stationarity was achieved, in order to identify the posterior probability tree set.

All 18S phylogenies produced from parsimony, likelihood, and Bayesian analyses were rooted with the sequence for *Coprinus* (a basidiomycete). *Coprinus* was chosen because it is part of the sister group to the ascomycetes and could be easily aligned with our endophyte sequences.

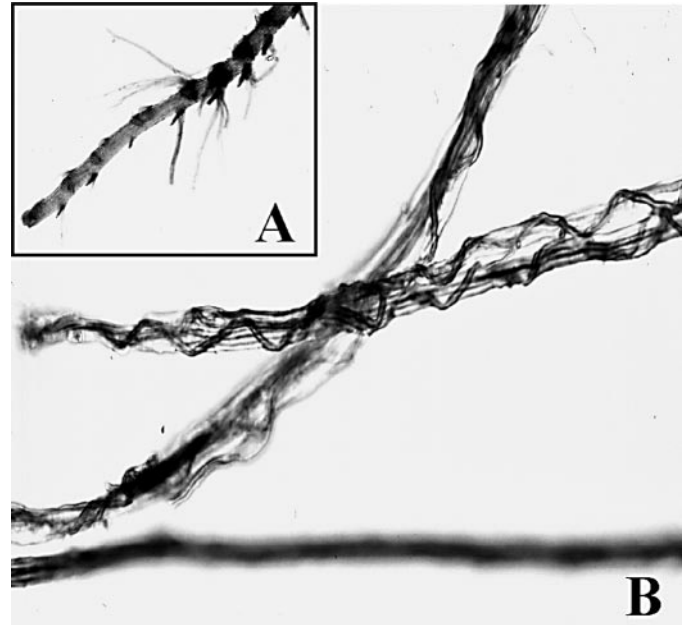


Fig. 1. (A) A penetrating stolon from *Bazzania*, showing rhizoids (40 $\times$ ). (B) Closer magnification, showing hyphae growing within and coiled around rhizoids (1000 $\times$ ).

## RESULTS

**Infection morphology and cultures**—Fungi were coiled around and growing within the rhizoids of *Bazzania*, but were not seen penetrating the thallus (Fig. 1). Nearly every rhizoid was infected. In *Odontoschisma*, fungi were seen clustered within the tips of nearly all rhizoids, but were also not observed penetrating the thallus. *Calypogeia* and *Metzgeria* showed a similar pattern of tip-clustered fungal infection, as in *Odontoschisma*. No rhizoids were present on *Plagiochila* or *Trichocolea*, and no fungal hyphae were visible using light microscopy.

Mycelia were successfully isolated for 13 out of 20 *Bazzania* specimens. Of the 13 cultures, four plates contained two mycelial morphotypes. These mixed cultures were subcultured, yielding a total of 17 pure cultures. Cultures were also isolated from two collections of *Odontoschisma*; of these, one contained more than one mycelium type, yielding three pure subcultures. *Calypogeia* yielded one pure culture. *Plagiochila*, *Metzgeria*, and *Trichocolea* each yielded one pure culture.

**Molecular analyses**—The ITS and 18S sequences were obtained from all cultures except one *Bazzania* isolate. The total number of sequences being compared for endophytes of *Bazzania* is thus 16. Amplification of 18s was unsuccessful for *Plagiochila* and *Trichocolea* cultures; results of only ITS data are therefore presented for these specimens.

**The ITS sequence similarity searches**—Based on BLAST results from ITS sequences, all of the endophytes isolated from *Bazzania* were inferred to belong to the Xylariales (see Supplementary Data accompanying the online version of this article for detailed BLAST results); 14 were closely matched to sequences from Xylariaceae. Of these xylariaceous endophytes, 10 were closely matched to sequences from *Xylaria*. Endophytes from two *Odontoschisma* cultures were also close-

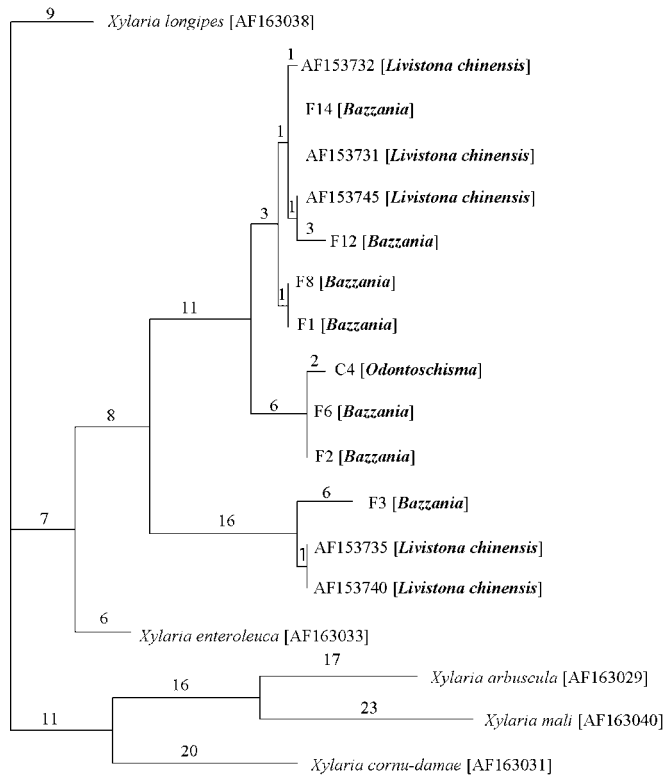


Fig. 2. Phylogenetic similarity of endophytes from *Bazzania* and from *Odontoschisma*, and endophytes from *Livistona chinensis* based on ITS sequences. Parsimony tree shown is one of two most parsimonious trees obtained from a branch and bound search. Numbers above branches indicate the number of nucleotide substitutions. Tree is intended only to show the similarity among endophytes isolated from different hosts, not to infer species relationships.

ly matched to sequences from Xylariaceae, one of which returned the same list of BLAST results as six of the *Bazzania* isolates. All endophytes from *Metzgeria*, *Plagiochila*, and *Trichocolea* were closely matched to sequences from *Xylaria*. The identity of one *Odontoschisma* isolate could not be determined using similarity searches, because the top matches were unidentified fungi. The *Calypogeia* isolate was closely matched to sequences from the Hypocreaceae.

**Phylogenetic analyses**—Ten ITS sequences from cultured endophytic fungi (nine *Bazzania*, one *Odontoschisma*) returned very close BLAST matches for xylariaceous endophytes cultured from angiosperms (Guo et al., 2000). Two of these (from *Bazzania*) could not be evaluated further using ITS, because the BLAST hits were “unidentified xylariacean endophyte(s)” (AF153741, AF153742, AF153743). A data matrix was compiled including the remaining eight liverwort endophyte sequences and their closely matched endophyte sequences from GenBank. Five additional non-endophyte *Xylaria* sequences were included in order to provide a phylogenetic context for the endophytes. Parsimony searches on this matrix resulted in two most parsimonious trees (68 parsimony-informative characters, consistency index [CI] = 0.77). All endophytes clustered together, and liverwort and angiosperm sequences were separated by only 1–7 nucleotide substitutions at the tips of the tree (Fig. 2). The tree shown is intended only to show the similarity among endophytes, not to infer relationships among species of *Xylaria*.

Based on ITS similarity results, we determined that the endophytic fungi from all six liverwort genera were pyrenomyces. In order to verify and further resolve identifications, a data matrix containing 18S sequences for 21 of the cultures and 40 from GenBank was constructed for phylogenetic analyses. The 40 GenBank samples included the major groups of pyrenomyces, a lecanoromycete, and a pezizomycete (Lutzoni et al., 2001).

Parsimony searches conducted on 18S data resulted in 31 000 most parsimonious trees (318 parsimony-informative characters, CI = 0.80). The maximum likelihood search yielded one tree. The Bayesian analysis reached stationarity at 441 000 generations, resulting in a total of 5600 trees in the posterior probability distribution. The strict consensus parsimony tree, the likelihood tree, and the 95% majority rule Bayesian tree were congruent and differed only slightly in their topologies. The Bayesian tree with posterior probability confidence values and parsimony bootstrap support values is shown in Fig. 3.

The 18S phylogeny resolves the pyrenomyces as monophyletic with high support. The Hypocreaceae, Valsaceae, and Ophiostomataceae form well-supported clades, and the latter have some support as sister lineages. There is also support for a monophyletic Xylariales. The relationship among these three major clades is unresolved. Within the Xylariales, a clade composed of Amphisphaeriaceae and Hyponectriaceae is strongly supported by the Bayesian analysis, although within that clade the two families are not resolved. The Xylariaceae are not resolved as a monophyletic group based on our 18S sequences. A clade containing three *Xylaria* sequences, one *Poronia* and an *Anthostomella* sequence, and 14 of the liverwort endophyte sequences is well supported. This topology is consistent with previously published phylogenetic analyses of pyrenomyces (e.g., Kang et al., 2002).

Eleven of the *Bazzania* endophyte sequences group with high support in the *Xylaria*/*Poronia*/*Anthostomella* clade. One *Metzgeria* and two *Odontoschisma* endophytes also fall within this group. One of the endophytes isolated from *Odontoschisma* is sister to an endophyte isolated from *Bazzania* (C8 and F3, respectively). One isolate from *Bazzania* (F16) is strongly supported as sister to a *Daldinia* (*Xylariaceae*) sequence obtained from GenBank. One *Odontoschisma* endophyte (C6), which could not be identified based on ITS, is highly supported as a close relative of the Ophiostomataceae. The fungus from *Calypogeia* (C1) is strongly supported as a member of the Hypocreaceae.

Relationships of two *Bazzania* endophytes (F15 and F17) are ambiguous, but they clearly belong within the Xylariales and are closely related to one another (Fig. 3). They share a large insertion of 140 nucleotides (nt) 530 nt from the 5' end (not included in phylogenetic analyses). This insertion is also present in F16 and the *Hypoxylon haematostroma* sequence, but is absent from all other sequences in this analysis. It is not present in the *Daldinia fissa* sequence obtained from GenBank, although that sequence is closely related to F16 on the basis of nucleotide substitutions. Based on these data, it seems likely that *H. haematostroma*, *Daldinia fissa*, and the two *Bazzania* isolates are closely related to one another, but their inter-relationships are not resolved by this analysis.

## DISCUSSION

**Phylogeny of liverwort endophytes**—Little molecular phylogenetic attention has been given the Xylariaceae, a diverse

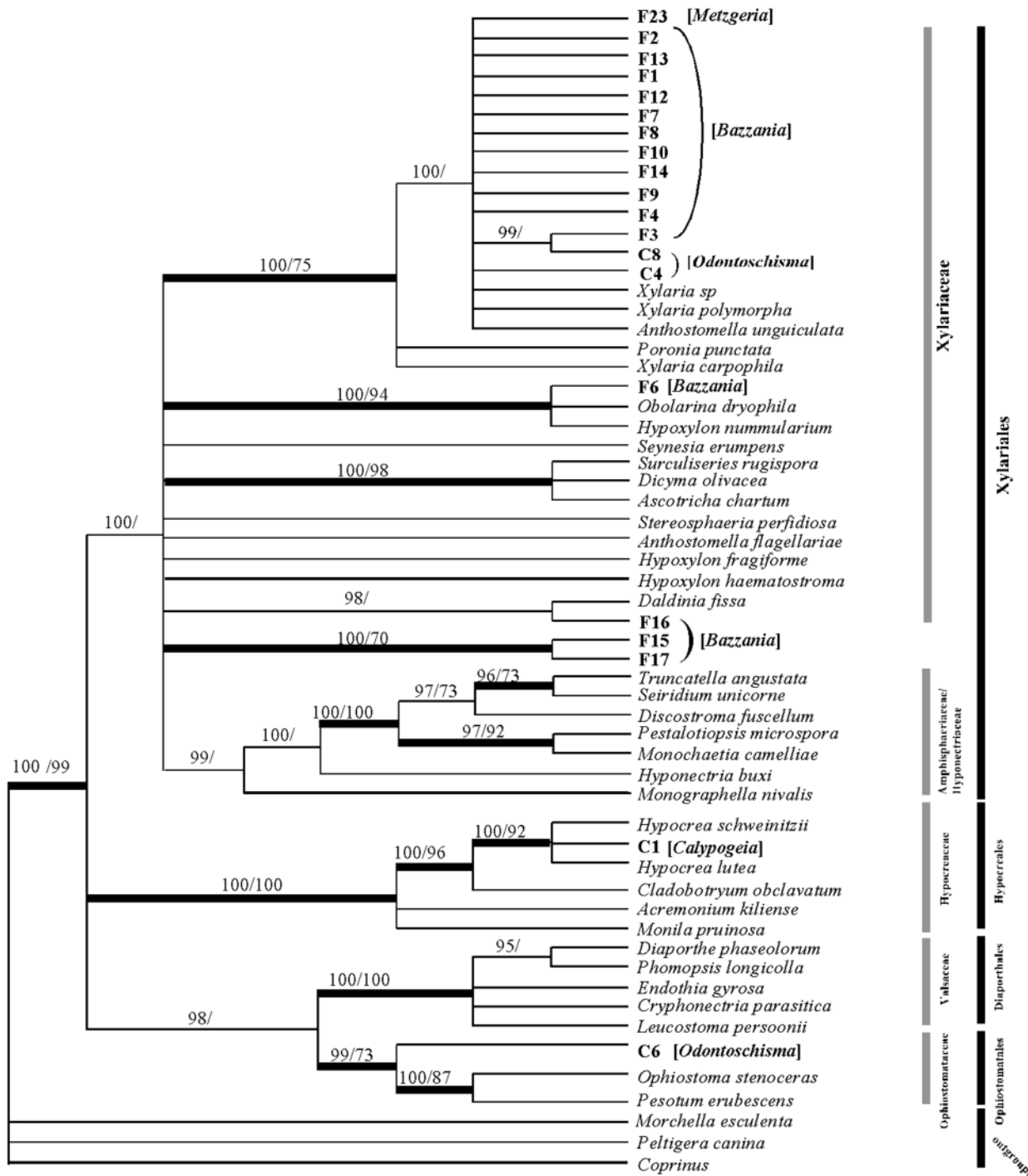


Fig. 3. Phylogenetic placement of endophytes from *Bazzania*, *Odontoschisma*, *Calypogeia*, and *Metzgeria* based on 18S sequences. Bayesian tree shown is the 95% majority rule of 4410 trees obtained from a 1000000 generation Markov chain Monte Carlo search. Numbers before slash indicate clade posterior probabilities of 95 or above. Numbers after slash indicate parsimony bootstrap support values of 70 or above. Branches leading to clades with both high probabilities and bootstrap support are in bold. Results from analyses rooted with *Coprinus* (a distant outgroup) were identical to those in which it was excluded and *Morchella* and *Peltigera* were used as outgroups.

group of ascomycetes. Lee et al. (2000) presented a phylogenetic analysis of *Xylaria* ITS sequences (18 taxa, 12 *Xylaria*), but other studies including substantial xylariaceous taxa have focused on identifying endophyte sequences (e.g., Guo et al., 2000; Collado et al., 2002; this study). Results from this study support previous analyses and highlight the need for

further phylogenetic examination of this group. *Xylaria* may be nonmonophyletic: *X. cubensis* may be more closely related to *Daldinia* (Lee et al., 2000), and 18S sequences from *Rosellinia*, *Anthostomella*, and *Poronia* group within the *Xylaria* clade (Collado et al., 2002; this study). It appears that the Amphisphaeriaceae/Hyponectriaceae clade is most closely re-

lated to taxa that have been classified in the Xylariaceae (Kang et al., 2002; this study). In general, no published molecular analysis provides convincing evidence that Xylariaceae, or any of its genera, is monophyletic. Much further study is needed in this ecologically complex group and should include a broad sampling of both endophytic and nonendophytic taxa.

The results of these molecular analyses suggest that endophytic *Xylaria* in liverworts and angiosperms are closely related. The ITS sequences from four *Bazzania* isolates are nearly identical to three endophytic *Xylaria* from *Livistona chinensis* (Arecaceae); two *Bazzania* isolates and one *Odontoschisma* are nearly identical to each other (Fig. 2). Such a pattern is not unprecedented. In studies of endophytic *Xylaria* using isozyme electrophoresis, Brunner and Petrini (1992) found that 17 of 32 endophytic *Xylaria* from different hosts formed a unique cluster. Together, these results indicate the presence of a large group of closely related *Xylaria* that are endophytic, but that have very broad host ranges. At present, closely related or identical *Xylaria* isolates have been identified from hosts belonging to three plant divisions and two continents.

To examine the frequency and phylogeny of *Xylaria* as liverwort endophytes, we are currently conducting a survey of xylariaceous endophytes across the phylogenetic spectrum of liverworts.

**The role of *Xylaria* in endophytic symbioses**—One hypothesis for the role of endophytic *Xylaria* posits that the fungi are simply waiting for their host to senesce (or perhaps to hasten it), at which time they can begin decomposition of cell wall materials (Petrini et al., 1995; Whalley, 1996). Endophytes employing this strategy would have an advantage over competing saprophytes, having “claimed” the tissue before decomposition begins. Studies on endophytic *Biscogniauxia* (Xylariaceae) in living oak tissue have shown that the same species is present in higher abundance on decaying twigs (Collado et al., 2001). However, data from studies examining fungal species composition in plant tissue before and after senescence do not support this hypothesis for all *Xylaria*. In a study of *Schefflera* (Araliaceae), Laessle and Lodge (1994) found different species of *Xylaria* occurring in living as compared to decomposing leaves. Bayman et al. (1998) found different species of *Xylaria* in the living leaves of *Manilkara* (Sapotaceae) than in the fallen leaves. In some oak different species of *Xylaria* occur in living and dead twigs. In beech, the same species of *Xylaria* was isolated at much lower frequency in decaying branches compared to healthy tissue (Griffith and Boddy, 1990). One alternative explanation for the role of xylariae that can be isolated as endophytes, but are not found decomposing the host plant, is that the fungus alternates between host taxa: one within which it exists as a cryptic endophyte and another on which it is saprophytic or pathogenic (Rogers, 2000; J. D. Rogers, personal communication). Such a pattern of host switching is seen in *Nemania serpens* (Carroll, 1999) and is thought to be common in *Xylaria* (Rogers, 2000). This lifestyle has been categorized as “foraging” (Carroll, 1999). Another explanation is that the endophytes exist only as endophytes, having become specialized to this environment (Rogers, 2000; J. D. Rogers, personal communication).

Comparative studies involving liverworts and their xylariaceous fungi have not been performed, thus it is unknown whether endophytic *Xylaria* also serve as decomposers of the host tissue. However, their endophytes may likely be foragers

or endophytic specialists because liverworts have no vascular tissue, thus they possess no wood and little cellulose to decompose compared to other plants; further, only a single record exists of a xylariaceous fungus producing fruiting bodies on liverwort substrate (Oudemans, 1919). Finally, endophytic *Xylaria* isolated from liverworts in this study appear to be more closely related to endophytes isolated from other plants than they are to saprophytic species.

**Could some *Xylaria* be mutualists?**—Endophytic *Xylaria* have several characteristics that are associated with mutualism and not latent saprophytism. Based on a review of empirical studies of antagonistic interactions between endophytes and grazers, insects, and microbial pathogens, Carroll (1988) outlined five general characteristics of endophyte mutualisms: (1) the endophyte is ubiquitous in a given host, geographically widespread, and causes minimal disease symptoms in the host plant; (2) vertical transmission or efficient horizontal transmission of the fungus occurs; (3) the fungus grows throughout host tissue, or, if confined to a particular organ, a high proportion of such organs are infected; (4) the fungus produces secondary metabolites likely to be antibiotic or toxic; and (5) the endophyte is taxonomically related to known herbivore or pathogen antagonists. Each of these characteristics as they apply to *Xylaria* are addressed in the following sections.

**Host specificity and geographic range**—Endophytic *Xylaria* occur on a broad diversity of plant hosts. Species delimitation based on cultures of endophytic *Xylaria* is difficult because of a lack of diagnostic characters. However, *Xylaria* have been isolated from *Euterpe*, *Trachycarpus*, and *Livistona* (Arecaceae; Rodrigues, 1994; Taylor et al., 1999; Guo et al., 2000); *Quercus* and *Fagus* (Fagaceae); *Betula*, *Corylus*, and *Alnus* (Betulaceae); *Acer* (Sapindaceae); *Fraxinus* (Oleaceae); *Rhizophora* and *Bruguiera* (Rhizophoraceae); *Avicennia* (Avicenniaceae); *Pinus* and *Picea* (Pinaceae); and *Nicotiana* (Solanaceae) (Brunner and Petrini, 1992); *Manilkara* (Sapotaceae; Lodge et al., 1996; Bayman et al., 1998); *Lepanthes* (Orchidaceae; Bayman et al., 1997); *Casuarina* (Casuarinaceae; Bayman et al., 1998); *Schefflera* (Araliaceae; Laessle and Lodge, 1994); *Heisteria* (Olacaceae) and *Ouratea* (Ochnaceae) (Arnold et al., 2000); and liverworts (present study). In addition, the group of endophytic *Xylaria* identified in this study appears to be cosmopolitan in their distribution. Endophytic *Xylaria* have also been isolated from vascular plants in Europe (Brunner and Petrini, 1992; Taylor et al., 1999), Malaysia (Brunner and Petrini, 1992), the Brazilian Amazon (Rodrigues, 1994), Puerto Rico (Laessle and Lodge, 1994; Lodge et al., 1996; Bayman et al., 1997, 1998), China (Taylor et al., 1999; Guo et al., 2000), Japan (Brunner and Petrini, 1992), and Panama (Arnold et al., 2000). Nearly identical ITS sequences ( $\leq 3\%$  divergent) were obtained from liverworts collected in Jamaica, North Carolina, and published sequences from China (Guo et al., 2000).

**Dispersal and transmission of endophytes**—There is some evidence that *Xylaria* can be vertically transmitted through seeds as in other mutualistic endophytes: *Xylaria* was reported in seeds of *Casuarina* (Bayman et al., 1998). However, given their global range, horizontal transmission by conidia or spores must also be effective.

**Tissue specificity and abundance of infection sites**—Endophytic *Xylaria* show moderate tissue specificity within their host plant. Some appear to be restricted to bark (Griffith and Boddy, 1990), while others are found primarily within vascular tissue or in the leaf veins (Rodrigues, 1994). In this study, endophytes were found in only the rhizoids of some of the liverworts. This pattern of endophyte infection has often been reported in hepatics (Pocock et al., 1984; Duckett and Read, 1991; Duckett et al., 1991; Williams et al., 1994; Duckett and Read, 1995; Chambers et al., 1999). In most culture studies of leaf endophytes, *Xylaria* is abundant in plant tissue (e.g., Rodrigues, 1994), and in this study of plants with rhizoids, fungi were seen in nearly every rhizoid examined. Further examination using staining techniques is needed to fully address the question of tissue specificity in liverwort endophytes.

**Secondary metabolites and related pathogen antagonists**—The production of secondary compounds that are toxic to herbivores or pathogens is a common characteristic of many endophytic mutualisms and also provides the basis for selection favoring the symbiosis in the host plant (Carroll, 1988). In vitro studies of endophytic *Xylaria* have shown that they actively produce secondary metabolites (Brunner and Petrini, 1992), and these may also be produced when the fungus inhabits living plant tissues. Such metabolites include antifungal and antibiotic compounds (Brunner and Petrini, 1992; Petrini et al., 1995). The secondary compounds of the xylariaceous endophyte, *Muscodor albus*, were experimentally shown to inhibit the growth of a broad range of plant and human pathogenic bacteria and fungi (Strobel et al., 2001). There has been no research on how these important compounds may affect host ecology.

Accumulating evidence suggests that relationships between endophytic *Xylaria* and their hosts are complex. Much further study of endophytic *Xylaria* is needed to fully understand their ecology. Transplant and inoculation experiments are also needed to address the question of whether *Xylaria* is a mutualistic, antagonistic, or commensalistic endophyte. We are currently attempting to conduct inoculation experiments with liverworts and their endophytic *Xylaria* in order to examine the effect of the fungus on host fitness.

Endophytes in the Hypocreales and the Ophiostomatales were also found growing within liverworts in this study. These fungi are well known for their interactions with vascular plants, fungi, and insects (e.g., *Claviceps* and *Cordyceps*, *Ophiostoma* and *Fusarium*). Their detection within liverworts is an intriguing area for future examination.

**Possible ecological links between vascular plants, fungi, and liverworts**—Duckett and Read (1995) suggested that the same species of ascomycetous fungi that forms ericoid mycorrhizae can also be found in the rhizoids of liverworts. They were able to synthesize the ericoid-type mycorrhiza in axenic plants using inoculum from liverworts. The combined results of this and the present study indicate that liverworts and angiosperms may serve as alternative hosts for particular fungi. Further, if endophyte links between these plants occur in nature, the potential for nutrient exchange and recycling among plants exists. The possibility of such a complex ecological web invites further study.

**Patterns in liverwort–fungal associations**—It is unclear whether liverwort endophytes are species- or habitat-specific.

While endophytes in *Cephaloziella exiliflora* appear to be the same in Antarctica and Australia (Chambers et al., 1999), different ascomycetes were isolated from *Calypogeia mulleriana* in the UK (Duckett and Read, 1995) and North America (present work). In addition, an hepatic-specific ascomycetous endophyte, *Mniaecia jungermanniae* (Nees ex Fr.) Boud. (Leotiaceae), has been documented from numerous liverworts, including *C. mulleriana* (Raspe and De Sloover, 1998). Results of the present study indicate that multiple endophytes infect the same liverwort individual and are suggestive that the same species of *Xylaria* and/or its close relatives have a wide host range. We are currently examining geographic patterns of endophyte diversity in another widespread temperate liverwort, *Scapania undulata* (L.) Dum. (Scapaniaceae).

**Evolution of the fungus–land plant association**—It has been suggested that the evolution of the fungus–plant mutualism was a crowning event in the evolutionary history of these two groups of eukaryotes, enabling them to colonize and dominate terrestrial habitats (e.g., Pirozynski and Malloch, 1975). The relationship between liverworts and *Xylaria* is likely to be a relatively new one, because although liverworts are one of the basal-most lineages of land plants (Nickrent et al., 2000), the clade containing *Xylaria* is more recently derived (Berbee and Taylor, 2001). Nevertheless, the morphology of their association may be suggestive of what these early plant–fungal associations looked like. Additional liverwort–fungal associations deserve further examination. For instance, the complex thalloid liverworts (Marchantiidae) reportedly contain endophytic Glomales (Boullard, 1988), which indeed may have evolved during the period when plants were invading land.

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