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Contributions of North American endophytes to the phylogeny, ecology, and taxonomy of Xylariaceae (Sordariomycetes, Ascomycota)

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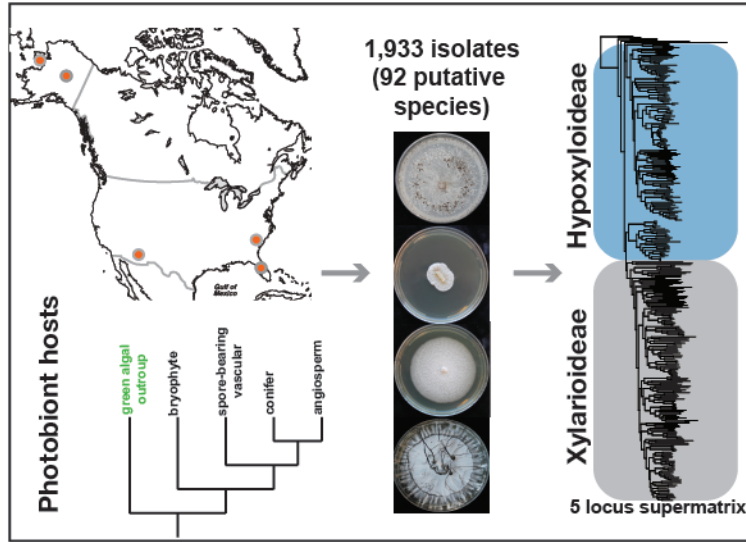
- Endophytes illuminate Xylariaceae circumscription and phylogenetic structure.
- Endophytes occur in lineages previously not known for endophytism.
- Boreal and temperate lichens and non-flowering plants commonly host Xylariaceae.
- Many have endophytic and saprotrophic life stages and are widespread generalists.

1 **Contributions of North American endophytes to the phylogeny,**
2 **ecology, and taxonomy of Xylariaceae (Sordariomycetes,**
3 **Ascomycota)**

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Abstract

The Xylariaceae (Sordariomycetes) comprise one of the largest and most diverse families of Ascomycota, with at least 85 accepted genera and ca. 1,343 accepted species. In addition to their frequent occurrence as saprotrophs, members of the family often are found as endophytes in living tissues of phylogenetically diverse plants and lichens. Many of these endophytes remain sterile in culture, precluding identification based on morphological characters. Previous studies indicate that endophytes are highly diverse and represent many xylariaceous genera; however, phylogenetic analyses at the family level generally have not included endophytes, such that their contributions to understanding phylogenetic relationships of Xylariaceae are not well known. Here we use a multi-locus, cumulative supermatrix approach to integrate 92 putative species of fungi isolated from plants and lichens into a phylogenetic framework for Xylariaceae. Our collection spans 1,933 isolates from living and senescent tissues in five biomes across North America, and here is analyzed in the context of previously published sequence data from described species and additional taxon sampling of type specimens from culture collections. We found that the majority of strains obtained in our surveys can be classified in the hypoxyloid and xylaroid subfamilies, although many also were found outside of these lineages (as currently circumscribed). Many endophytes were placed in lineages previously not known for endophytism. Most endophytes appear to represent novel species, but inferences are limited by potential gaps in public databases. By linking our data, publicly available sequence data, and records of ascomata, we identify many geographically widespread, host-generalist clades capable of symbiotic associations with diverse photosynthetic partners. Concomitant with such cosmopolitan host use and distributions, many xylariaceous endophytes appear to have both endophytic and saprotrophic life stages. Overall, our study reveals major gaps in the availability of multi-locus datasets and metadata for this iconic family, and provides new hypotheses regarding the ecology and evolution of endophytism and other trophic modes across the family Xylariaceae.

1. Introduction

Fungi are one of the most diverse and ecologically important clades of life (Hammond, 1995; Agrios, 2005), yet only a tiny fraction of their estimated diversity has been discovered and described (i.e., < 5%; Hawksworth, 1991; Hawksworth, 2001; Mueller and Schmit, 2007). Many of the 'missing fungi' – species filling the gap between the number thought to exist (1.5 to 9 million species, Cannon, 1997; Hawksworth, 1991; Hawksworth, 2001; O'Brien et al., 2005) and those described to date (ca. 99,000 species; Blackwell, 2011) – are microfungi living in cryptic symbioses (Agrios, 2005; Blackwell, 2011; Hawksworth, 1991; Hyde, 2001). In particular, an enormous amount of yet-unknown diversity is thought to occur as endophytes, which inhabit apparently healthy, above-ground tissue of all major lineages of land plants (i.e., Class 3 endophytes, sensu Rodriguez et al., 2009), and as endolichenic fungi, which occur in symptomless lichen thalli in close association with the photobiont (i.e., the algal or cyanobacterial partner in lichen thalli; Arnold et al., 2009; Li et al., 2007; Suryanarayanan et al., 2005; U'Ren et al., 2012; U'Ren et al., 2014).

The majority of foliar endophytes and endolichenic fungi (hereafter, globally referred to as endophytes) are members of the Pezizomycotina (see Rodriquez et al., 2009), with particular diversity in the Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes, and Sordariomycetes (e.g., Arnold et al., 2009; Bálint et al., 2015; Chen et al., 2015; Davey et al., 2013; Tedersoo et al., 2013; U'Ren et al., 2012; U'Ren et al., 2014; Wang et al., 2006; Zimmerman and Vitousek, 2012). These endophytes occur in ecosystems ranging from hot deserts to wet forests, to arctic tundra (e.g., Arnold et al., 2009; Davis et al., 2003; Del Olmo-Ruiz and Arnold, 2014; Gazis and Chaverri, 2010; Higgins et al., 2007; Massimo et al., 2015; Suryanarayanan et al., 2011; U'Ren et al., 2012; Zimmerman and Vitousek, 2012). Individual plants and lichen thalli can harbor phylogenetically diverse endophytes, with significant turnover across the geographic ranges of their hosts (e.g., Fisher et al., 1994; Fisher et al., 1995; Higgins et al., 2014; U'Ren et al., 2012; Vaz et al., 2014; Zimmerman and Vitousek, 2012). Endophytes in plants can play important ecological roles, mediating defense against pathogens and herbivores

105   and influencing host responses to abiotic stressors such as drought (Arnold et al., 2003; Arnold

106   and Engelbrecht, 2007; Costa Pinto et al., 2000; Estrada et al., 2013; Mejía et al., 2008). They

107   are increasingly recognized as a major source of novel metabolic products for use in medicine,

108   agriculture, and industry (Ding et al., 2009; Deyrup et al., 2007; Fan et al., 2014; Jiménez-

109   Romero et al., 2008; Paranagama et al., 2007; Staniek et al., 2008; Strobel et al., 1997; Strobel

110   and Long, 1998; Wu et al., 2011) and as an important but under-studied aspect of plant and

111   lichen biology.

112   Studies over the last four decades have revealed an extremely high richness of

113   xylariaceous endophytes (Xylariales, Sordariomycetes, Pezizomycotina) (e.g., Petrini and Petrini,

114   1985; Rogers, 2000). They inhabit the living tissues of phylogenetically diverse plants, including

115   conifers, angiosperms, ferns, lycophytes, and bryophytes in a diverse range of biogeographic

116   provinces (e.g., Arnold et al., 2009; Brunner and Petrini, 1992; Carroll and Carroll, 1978; Davey et

117   al., 2014; Davis et al., 2003; Del Olmo-Ruiz and Arnold, 2014; Higgins et al., 2007; Okane et al.,

118   2012; Petrini and Petrini, 1985; Petrini et al., 1995). They also occur frequently in taxonomically

119   diverse lichens encompassing diverse growth forms (e.g., foliose, fruticose, crustose) and

120   substrates (e.g., terricolous, saxicolous, epiphytic) (Arnold et al., 2009; Petrini and Petrini, 1985;

121   Suryanarayanan et al., 2005; Wu et al., 2011). Some xylariaceous endophytes persist in leaf

122   litter, reflecting abilities to decompose lignocellulose and the capacity of some species to directly

123   infect decaying leaves, potentially circumventing the need for an endophytic life stage (Osono,

124   2002; 2005; 2006).

125   Many endophytic Xylariaceae remain sterile in culture or reproduce only asexually on

126   standard media (Stadler et al., 2013), precluding identification based on teleomorphic characters

127   such as stromata features and ascospore number (see Petrini and Petrini, 1985; Rogers, 1979a;

128   2000; Rogers et al., 2002). Anamorphic cultures can be classified based on conidiophore

129   branching and the nature of conidiogenous cell proliferation (Ju and Rogers, 1996), as well as

130   cultural characteristics such a growth rates and color (Petrini and Petrini, 1985). However,

131   anamorphic characters alone often lack sufficient information for species-level identification

132   (Petrini and Petrini, 1985; Stadler et al., 2013). As a result, estimates of species boundaries and

133 taxonomic placement of endophytes frequently are assigned on the basis of BLAST comparisons

134 of barcode sequences (i.e., nuclear ribosomal internal transcribed spacers and 5.8S; ITS rDNA)

135 in GenBank. This approach is often problematic due to inconsistencies in levels of interspecific

136 ITS rDNA variation among taxonomic groups (Nilsson et al., 2008), misidentified sequences in

137 public databases (Bridge et al., 2003; Harris, 2003; Peršoh et al., 2009; Vilgalys, 2003), and

138 problems posed by under-representation of fungal biodiversity in public databases, such that the

139 closest named BLASTn hit is not always the closest relative (see Gazis et al., 2012; U'Ren et al.,

140 2009). Misassignment of unknown sequences can erroneously expand or contract the family

141 concept over time, alter taxonomic concepts for genera and species (e.g., *Xylaria hypoxylon*; see

142 Peršoh et al., 2009), and confound ecological inferences for newly discovered strains for which

143 only ITS rDNA or other single-locus data are available.

144   As currently circumscribed, Xylariaceae comprise one of the largest and most diverse

145 families of filamentous Ascomycota, with at least 85 accepted genera and an estimated 1,343

146 accepted species (Eriksson, 2006; Kirk et al., 2008; Stadler et al., 2013). Traditionally two

147 subfamilies are recognized: Hypoxyloideae and Xylarioideae (Dennis, 1961). Currently

148 recognized species include saprotrophs occurring in wood, litter, soil, and dung, and a few plant

149 pathogens that cause canker diseases (e.g., *Entoleuca mammata* on *Populus*), root rots (e.g.,

150 *Xylaria mali* on *Malus*), and needle blight (e.g., *Hypoxylon herpotrichoides* on *Pseudotsuga* and

151 *Picea*) in agricultural and natural systems (Edwards, 2003; Martin, 1967; Rogers, 1979a; 2000;

152 Rogers and Ju, 1996; Whalley, 1985). Although several described species are close matches

153 with endophytes in BLAST comparisons of ITS rDNA sequences, the taxonomic placement of

154 those endophytic isolates rarely is investigated based on multi-locus phylogenetic analyses (but

155 see Bills et al., 2012; Pažoutová et al., 2010b; Visser et al., 2009). To our knowledge, few novel

156 species of Xylariaceae have been described solely as endophytes from anamorphic cultures (but

157 see Worapong et al. [2001] and González et al. [2009] for description of *Muscodor* spp.).

158   Given the captivating ascomata morphologies (i.e., spore-bearing structures resulting

159 from sexual reproduction) and ecological importance of *Xylaria* and related taxa, the Xylariaceae

160 have long received attention from mycologists. A particularly rich tradition of morphological

161 systematics (e.g., Dennis, 1957; Hawksworth and Whalley, 1985; Ju et al., 1993; Ju and Rogers,

162 1996; Ju et al., 1998; Læssøe et al., 1989; Miller, 1961; Möller, 1901; Pouzar, 1985a; 1985b;

163 Rogers, 1981; Rogers and Ju, 1996; Rogers et al., 1997a; 1997b) is increasingly complemented

164 by chemotaxonomic (e.g., Fournier et al., 2010; Læssøe et al., 2010; 2013; Stadler and Fournier,

165 2006; Stadler et al., 2008; 2010) and molecular approaches (e.g., Bills et al., 2012; Hsieh et al.,

166 2005; 2010; Jaklitsch et al., 2014; Peršoh et al., 2009; Whalley, 1996). Several genera have been

167 updated or monographed recently (e.g., Daranagama et al., 2015; Rogers et al., 2002; Peršoh et

168 al., 2009; Stadler et al., 2014) and NCBI contains nucleotide data for >3,000 isolates representing

169 ca. 442 recognized species (as of August 2015). Phylogenetic analyses support monophyly of the

170 family as presently circumscribed (Tang et al., 2009), but numerous studies suggest that many

171 recognized genera and species may not be monophyletic (see Daranagama et al., 2015; Hsieh et

172 al., 2005; 2010; Pažoutová et al., 2010b), and many genera require taxonomic revisions (Stadler

173 et al., 2013). Integrating previously unknown strains into a robust phylogenetic framework for the

174 Xylariaceae can provide insight into the taxonomic circumscription at the family and infrafamilial

175 levels, illustrate previously unknown connections between anamorphic and teleomorphic species,

176 and inform evolutionary relationships, host ranges, distributions, major ecological modes, and

177 diversity of major clades, previously known taxa, newly found strains, and the family as a whole.

178   The goal of this study was to address the impact of a large collection of endophytic and

179 saprotrophic fungi on the circumscription of the Xylariaceae and infrafamilial taxa. Our work takes

180 advantage of 1,933 newly cultured isolates representing 92 putative species, which were

181 collected from living photosynthetic tissues of angiosperms, conifers, ferns, lycophytes,

182 bryophytes, and lichens, as well as senescent and decomposing leaves of selected woody plants

183 in five biomes across North America (U'Ren et al., 2010; 2012). Here, we place these strains in a

184 multi-locus phylogenetic framework in conjunction with additional sequencing of type specimens

185 from culture collections and previously published sequence data from described species. We then

186 address the following questions: (1) Does the inclusion of newly cultured isolates alter current

187 phylogenetic hypotheses regarding the delimitation of Xylariaceae and the relationships and

188 circumscription of xylariaceous taxa? (2) What is the classification of these isolates? (3) Do they

189   represent novel species or anamorphs of previously described teleomorph species? (4) How can

190   these cultures expand our knowledge of the host affiliations, substrate use, geographic

191   distribution, and phylogenetic diversity of the Xylariaceae? In addressing these questions we

192   provide an overview of currently available metadata for members of the Xylariaceae, and highlight

193   emergent patterns regarding ecological modes across this diverse, important, and

194   morphologically compelling family.

195

196   **2. Materials and Methods**

197

198   *2.1. Field surveys*

199

200   As part of a larger study investigating the diversity and distributions of endophytic fungi, living

201   leaves and healthy lichen thalli were collected systematically in five sites representing distinct

202   environmental, biological, and biogeographic regions across North America (U'Ren et al., 2012).

203   Sites were located in the Madrean Sky Island Archipelago of southeastern Arizona (AZC); the

204   Appalachian Mountains of western North Carolina (NCH); sub-tropical scrub forest in Florida

205   (FLA); Beringian tundra and boreal forest in the Seward Peninsula ecoregion of western Alaska

206   (AKN); and inland, subalpine tundra in the Interior Highlands of east-central Alaska (AKE).

207   Endophytes were cultured from surface-sterilized tissues of living, apparently healthy plants

208   (angiosperms, conifers, lycophytes, ferns, and bryophytes) and lichens (with diverse mycobionts,

209   substrates, and growth forms) as described in U'Ren et al. (2012). In each site fungi also were

210   cultured concurrently from surface-sterilized tissues of senescent leaves in the canopy of

211   selected woody plants (i.e., dead plant leaves, DP) and leaf litter (i.e., fallen leaves of the same

212   species, FP) (U'Ren et al., 2010; U'Ren, 2011). Classifying fungi broadly as "endophytes" or

213   "saprotrophs" based on the condition of the tissue from which they are isolated is insufficient to

214   adequately define their ecological roles (U'Ren, 2011). However, for the purposes of this study

215   fungal OTU isolated from living host tissues (either plant or lichen) are referred to as endophytes

216   (even if isolates were found in non-living tissues as well), whereas fungal OTU isolated only from

217 non-living plant tissues (i.e., DP and/or FP) are referred to as saprotrophs. Each isolate is

218 maintained as an axenic voucher in sterile water at the Robert L. Gilbertson Mycological

219 Herbarium at the University of Arizona (ARIZ) (Supplemental Table 1).

220

221 *2.2. Sequencing the ITS-partial LSU rDNA of field-collected strains*

222

223 Overall, the field surveys described above generated 6,784 cultures, which were screened by

224 DNA sequencing for preliminary taxonomic placement. Methods for DNA extraction, PCR

225 amplification, DNA sequencing and sequence editing followed U'Ren et al. (2010). Briefly, DNA

226 was extracted from each isolate using phenol:chloroform:IAA (Arnold and Lutzoni, 2007). The

227 nuclear ribosomal internal transcribed spacers and 5.8S (i.e., ITS rDNA) were amplified by PCR

228 with ca. 500 bp of the adjacent nuclear ribosomal large subunit (LSU rDNA) as a single fragment

229 using primers ITS1F/LR3 or ITS5/LR3 (Gardes and Bruns, 1993; Vilgalys and Hester, 1990;

230 White et al., 1990). Amplicons were sequenced bidirectionally with the above primers using

231 Applied Biosystems BigDye® Terminator v3.1 cycle sequencing kits (Applied Biosystems 3730*xl*

232 DNA Analyzer; Foster City, CA, USA) at the University of Arizona Genetics Core. The software

233 applications *phred* and *phrap* (Ewing and Green, 1998; Ewing et al., 1998) were used to call

234 bases and assemble contigs with automation provided by the ChromaSeq package in Mesquite

235 (Maddison and Maddison, 2011; http://mesquiteproject.org). Base calls were verified by visual

236 inspection of chromatograms in Sequencher v. 4.5 (Gene Codes, Ann Arbor, MI).

237   Sequences were assembled into groups by first generating a distance matrix in ESPRIT

238 (Sun et al., 2009) based on pairwise Needleman-Wunsch alignments for all sequence pairs with

239 *k*-mer distances less than 0.5, followed by clustering using the furthest neighbor algorithm in

240 mothur (Schloss et al., 2009). Groups were defined by 100%, 99%, and 95% sequence similarity

241 as a proxy for delimiting genotypes (100%, 99%) and putative species (95%) following U'Ren et

242 al. (2009) and Liggenstoffer et al. (2010). A single representative sequence for each 95%

243 similarity group (hereafter, operational taxonomic unit, OTU) was queried against the curated ITS

244 rDNA sequence database at the Alaska Fungal Metagenomics Project

245   (http://www.borealfungi.uaf.edu/) using BLASTn (Altschul et al., 1990) to estimate taxonomic

246   affiliation. Overall, 92 OTU and 245 unique genotypes (based on 95% and 100% sequence

247   similarity, respectively) had top BLASTn hits to taxa identified as Xylariaceae. These OTU

248   comprise a total of 1,933 isolates (Supplemental Table 1).

249

250   *2.3. Multi-locus sequencing of field-collected strains*

251

252   A single isolate from each 95% OTU (with the exception of one OTU with two isolates in different

253   99% OTU) was selected for morphological examination and multi-locus sequencing (Table 1).

254   The resulting set of 92 OTU included 39 OTU (131 isolates) found only in living plant tissues or

255   lichen thalli (i.e., endophytes), 44 OTU (1,780 isolates) found in both living tissues and dead or

256   fallen leaves (here, treated as endophytes), and 9 OTU (22 isolates) found only in dead or fallen

257   leaves (here treated as saprotrophs).

258   Based on previously published multi-locus studies of Hypoxyloideae (Hsieh et al., 2005)

259   and Xylarioideae (Hsieh et al., 2010), we focused on three protein-coding genes (β-tubulin, α-

260   actin and *RPB2*). β-tubulin and α-actin were amplified by PCR using primer pairs T1/T22 or

261   T11/T22 (O'Donnell and Cigelnik, 1997) and ACT-512F/ ACT-783R (Carbone and Kohn, 1999),

262   respectively. Approximately 1 kb of the gene encoding the RNA polymerase II second-largest

263   subunit (*RPB2*) was amplified with the primer pair fRPB2-5F/ fRPB2-7cR (Liu et al., 1999). Each

264   25 µl reaction contained a final concentration of 5 ng of genomic DNA, 0.6 µM forward and

265   reverse primers, 0.08 mg/ml Bovine serum albumin (BSA), and 1X REDTaq ® ReadyMix (Sigma-

266   Aldrich, St. Louis, MO, USA). Because the majority of samples (i.e., 63 out of 92) failed to amplify

267   using previously published PCR protocols for *RPB2* (Liu et al., 1999) we performed a two-step

268   touchdown PCR following U'Ren et al. (2007) for the remaining isolates. After initial denaturation

269   at 94° C for 5 min, 30 cycles of touchdown PCR were performed (denaturation at 94° C for 1 min,

270   annealing for 1 min with a 0.5° C/cycle decrement starting at 60° C, and an extension at 72° C for

271   1 min), followed by 20 cycles of regular PCR (95° C for 1 min, 45° C for 1 min, 72° C for 1 min,

272   and a final extension step for 5 min at 72° C). After an initial β-tubulin PCR with eight isolates

273   revealed no amplification with previously published protocols (O'Donnell and Cigelnik, 1997), the

274   two-step touchdown protocol was used to amplify all isolates. Negative controls, which contained

275   all components except DNA templates, were included in parallel.

276   PCR products were evaluated by staining with SYBR Green I (Molecular Probes,

277   Invitrogen, Carlsbad, CA, USA) after electrophoresis on a 1% agarose gel. When positive

278   amplicons yielded single bands, PCR products were sequenced directly as described below.

279   When isolates displayed multiple bands or weak amplification, PCR products were cloned using

280   the Strataclone PCR Cloning Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's

281   instructions, except that one-half the recommended reagent volumes were used for each

282   reaction. After blue/white screening, successfully transformed colonies were transferred to new

283   plates and incubated an additional 24 h to increase colony size. Five positive clones per isolate

284   were amplified in secondary PCR with primers M13F and M13R. Up to five amplicons per isolate

285   were selected for sequencing. PCR products were cleaned by adding 1 µl of ExoSAP-IT

286   (Affymetrix, Santa Clara, CA, USA) to 20 µl of PCR product and incubating for 60 min at 65°C

287   followed by 15 min at 85°C. Following a 1:2 dilution, PCR products (either directly from initial PCR

288   or from secondary PCR from cloning) were sequenced and edited as described above.

289   Bidirectional sequences were assembled and edited as described above. Edited

290   consensus sequences were queried against NCBI using BLASTn to estimate taxonomic

291   placement. Protein-coding sequences were subject to a BLASTx search to determine the reading

292   frame and the start/stop positions of each exon when applicable (i.e., β-tubulin and α-actin). All

293   sequences generated from cultures have been deposited in GenBank under accession numbers

294   XXXXX-XXXXX.

295

296   *2.4. Sampling of previously described taxa*

297

298   Available sequence data (as of January 2015) for ITS rDNA, LSU rDNA*,* β-tubulin, α-actin, and

299   *RPB2* were downloaded from NCBI or the AFToL database (www.aftol.org) for 293 species

300   (representing 429 accessions) of Xylariomycetidae. These species represent families of

301 Xylariales proposed by Smith et al. (2003) and revised by Senanayake et al. (2015), providing the

302 basis for establishing the family boundaries of Xylariaceae: Apiosporaceae (2 species),

303 Cainiaceae (1 species; see Jeewon, 2002), Diatrypaceae (32 species), Graphostromataceae (1

304 species), Hyponectriaceae (4 species), Lopadostomaceae (1 species), Pseudomassariaceae (1

305 species), Xylariaceae (208 species), as well as Xylariales *incertae sedis* isolates (6 species)

306 (Supplemental Table 2). Several putative members of Xylariales were not included due to a lack

307 of sequence data for protein-coding genes: Coniocessiaceae (García et al., 2006), Vialaeaceae

308 (Shoemaker et al., 2013), Melogrammataceae (see Senanayake et al., 2015), and

309 Iodosphaeriaceae (see Senanayake et al., 2015). Families previously classified within Xylariales,

310 but recently proposed for placement in the Amphisphaeriales, also were included

311 (Amphisphaeriaceae [33 species] and Clypeosphaeriaceae [4 species]; Supplemental Table 2;

312 see Senanayake et al., 2015). *Ophiostoma ulmi*, *O. piliferum*, and *O. stenoceras* were used to

313 root the tree following Huhndorf et al. (2004) and Tang et al. (2007).

314 In addition, 26 putative species of Xylariaceae were obtained from the Centraalbureau

315 voor Schimmelcultures (CBS) Fungal Biodiversity Centre (Utrecht, Netherlands; Supplemental

316 Table 2). Cultures were chosen to represent genera and species that were lacking molecular data

317 in public databases at the time (June 2011), focusing on major clades of Xylariaceae. On receipt,

318 cultures were immediately plated on 2% malt extract agar and grown at room temperature for 1-2

319 weeks. Once sufficient mycelium was present, DNA was extracted and ITS rDNA-partial LSU

320 rDNA, β-tubulin, α-actin, and *RPB2* were PCR-amplified and sequenced as described above.

321 Sequence data for these CBS isolates have been deposited in GenBank under accession

322 numbers XXXXX-XXXXX (Supplemental Table 2).

323

324 *2.5. Sequence alignment and topological incongruence tests*

325

326 ITS rDNA and ITS-partial LSU rDNA sequences were analyzed using Fungal ITS extractor

327 (Nilsson et al., 2010) to create separate fasta files for ITS1, 5.8S, ITS2, and LSU rDNA. LSU

328 rDNA sequences were aligned according to the secondary structure of *Saccharomyces*

329   *cerevisiae* as described in Miadlikowska et al. (2006). 5.8S sequences were aligned with

330   MUSCLE 3.8.31 (Edgar, 2004) using default parameters and then edited manually. Alignments

331   for protein-coding genes were first done at the amino acid level using MUSCLE as implemented

332   in Mesquite (Maddison and Maddison, 2011). Aligned amino acids for β-tubulin, α-actin and

333   *RPB2* were then back-translated to obtain nucleotide alignments and manually adjusted using the

334   "Nucleotide with AA color" option in Mesquite. For LSU and *RPB2*, ambiguously aligned

335   nucleotides (sensu Lutzoni et al., 2000) were delimited manually and excluded from subsequent

336   analyses (Supplemental Table 3). ITS1 rDNA, ITS2 rDNA, and introns from β-tubulin were too

337   divergent to be reliably aligned at such a broad taxonomic level; therefore, we tested the impact

338   of excluding these regions vs. including them in analyses after recoding as non-DNA characters

339   using Principal Coordinates Analysis (PCoA) as implemented in PICS-Ord (Lücking et al., 2011).

340   Preliminary maximum likelihood (ML) analyses were performed on a total of six single-

341   locus datasets (recoded ITS1, 5.8S, and recoded ITS2 rDNA; 5.8S rDNA; LSU rDNA; *RPB2*; β-

342   tubulin exons; and β-tubulin exons plus recoded introns) at the nucleotide level using

343   RAxMLHPC-MPI-SSE3 version 7.7.6 (Stamatakis, 2006) on the Mobyle SNAP Workbench

344   version 1.0.5 (Monacell and Carbone, 2014). Optimal tree and bootstrap searches were

345   conducted with the default rapid hill-climbing algorithm for 1000 replicates with GTR substitution

346   model (Rodríguez et al., 1990) and gamma distribution approximated with four categories in all

347   analyses. Recoded ITS1 rDNA, ITS2 rDNA, and introns from PICS-Ord were analyzed as

348   unordered characters with the GTR substitution model following recommendations in Lücking et

349   al. (2011). For each protein-coding gene, subsets of partitions were defined with the program

350   PartitionFinder v.1.1.0 (Lanfear et al., 2012), the greedy search option, and the Bayesian

351   information criterion (BIC) for model selection.

352   To identify topological incongruence among the resulting single-locus trees, a reciprocal

353   70% ML bootstrap support criterion was applied (Mason-Gamer and Kellogg, 1996). Briefly, a

354   conflict was considered significant if taxa in a strongly-supported monophyletic clade (i.e., with

355   ≥70% bootstrap value) based on one locus were strongly supported as non-monophyletic (i.e.,

356   ≥70% bootstrap value) in another single-locus tree. No significant conflict was detected between

357   single-locus tree topologies with and without recoded data (i.e., β-tubulin exons vs. β-tubulin

358   exons plus recoded introns; ITS1 rDNA, ITS2 rDNA recoded plus 5.8S rDNA vs. 5.8S rDNA only),

359   thus the single-locus alignments including the recoded data were concatenated into a single

360   supermatrix for phylogenetic analyses.

361

362   *2.6. Phylogenetic analyses to delimit Xylariaceae and place newly cultured strains within*

363   *Xylariomycetidae*

364

365   The following seven subsets (obtained with PartitionFinder) were analysed with RAxML as

366   described above to infer relationships within Xylariomycetidae: (1) 5.8S, β-tubulin first codon

367   position, and LSU rDNA; (2) *RPB2* first codon position; (3) *RPB2* second codon position; (4)

368   *RPB2* third codon position; (5) β-tubulin second codon position; (6) β-tubulin third codon position;

369   and (7) recoded ITS1 rDNA, ITS2 rDNA, and β-tubulin introns. The final concatenated dataset for

370   Xylariomycetidae containing 520 terminal taxa (including three *Ophiostoma* spp. as the outgroup)

371   has been deposited in TreeBASE (XXXXX). Sequences for α-actin were not included in these

372   analyses because they were not available in GenBank for Xylariomycetidae representatives other

373   than Xylariaceae.

374   Once the taxonomic boundaries for Xylariaceae were estimated using this concatenated

375   supermatrix for Xylariomycetidae (Supplemental Fig. 1), non-Xylariaceae taxa were removed

376   from each alignment (except *Diatrype disciformis* and *Eutypa lata,* which were chosen to root

377   subsequent trees based on the topology of the Xylariomycetidae phylogeny and the availability of

378   multi-locus data; Supplemental Fig. 1; Supplemental Table 2). Although preliminary analyses

379   placed *Graphostroma platystoma* (the sole species in Graphostromataceae; Barr et al., 1993) in a

380   well-supported clade with *Biscogniauxia arima, B. marginata, B. granmo,* and *B. simplicior* (a

381   placement that agrees with morphological similarity between anamorphs of *G. platystoma* and

382   hypoxyloid Xylariaceae), it was removed due to uncertainty regarding its affinities to Xylariaceae

383   (see Senanayake et al., 2015) and low quality sequence data for non-ribosomal loci (see

384   Supplemental Table 2). Additionally, isolates considered previously to be within the Xylariaceae,

385   but which appeared outside of the monophyletic Xylariaceae in these analyses, also were

386   removed (i.e., *Anthostomella torosa*, *Dicyma funiculosa*, *D. pulvinata*, and 13 newly isolated OTU

387   tentatively identified as Xylariaceae based on BLASTn; Supplemental Tables 1-2; Table 1).

388   Because analyses were performed prior to the reclassification of *Seynesia* in the Cainaceae (see

389   Senanayake et al., 2015), these analyses included *Seynesia erupens* as well as two endophyte

390   OTU (clades E1-E2) that are potentially outside Xylariaceae. As described below, their resulting

391   placement is not in conflict with recent studies (see Senanayake et al., 2015).

392

393   *2.7. Phylogenetic analyses of Xylariaceae using a cumulative supermatrix approach*

394

395   When taxon sampling was narrowed to focus on putative Xylariaceae, a total of 79 putative

396   species defined at 95% ITS-partial LSU rDNA sequence similarity from our collections

397   (representing 1,815 isolates total) remained in the analysis. The family-level focus decreased the

398   prevalence of ambiguous regions in the *RPB2* and LSU rDNA alignments, and alignments were

399   adjusted to gain additional phylogenetically informative characters (Supplemental Table 3). For

400   each individual locus, ML analyses and assessment of topological incongruence were performed

401   as described above. No significant conflict was detected between single locus tree topologies

402   with and without recoded data, such that all recoded data were kept in the concatenated dataset.

403   However, conflict among different loci resulted in the removal of seven taxa (FL0975, NC1612,

404   *Nemania diffusa* AT-113, *N. aenea* JF02118, *N. serpens* AT-114, *N. chestersii* JF04024, and

405   *Xylaria* sp. XT09003). For four additional taxa, single sequences that were in conflict with other

406   loci were removed (*RPB2* FL0933; β-tubulin FL0016, FL0804, and *Xylaria escharoidea* 658;

407   Supplemental Table 4). Conflicting sequences potentially represent alternative copies of β-tubulin

408   (see Keeling et al., 2000; Landvik et al., 2001) or contaminants. After removing conflicting

409   sequences single-locus analyses were repeated to assess congruence.

410   Following the assessment of congruence, the single-locus alignments were concatenated

411   into a single supermatrix for subsequent ML analysis. The supermatrix contained 77 putative

412   species (representing 1,778 isolates total and 78 terminal taxa) from our collections as well as

413 209 previously described taxa. DNA partitions for the five-locus supermatrix were analyzed in

414 PartitionFinder using the parameters described above. The following seven subsets were

415 specified for the RAxML analysis: (1) α-actin first codon position, α-actin second codon position,

416 and β-tubulin second codon position; (2) third codon position for both α-actin and β-tubulin; (3)

417 5.8S rDNA, α-actin introns (for which 15 bp could be reliably aligned), β-tubulin first codon

418 position, and LSU rDNA; (4) *RPB2* first codon position; (5) *RPB2* second codon position; (6)

419 *RPB2* third codon position; and (7) recoded β-tubulin introns, α-actin introns, ITS1 rDNA and ITS2

420 rDNA. The final concatenated alignment containing 367 terminal taxa has been deposited in

421 TreeBASE (XXXXX).

422   Because only a subset of taxa within Xylariaceae were represented by all five loci (Table

423 1; Supplemental Table 2), we examined the effect of adding taxa with an increasing amount of

424 missing data using a cumulative supermatrix approach (following Miadlikowska et al. 2006; 2014;

425 and Gaya et al., 2012). Four individual datasets were analyzed: (1) taxa containing a minimum of

426 four loci (i.e., taxa with 5 loci sequenced + taxa with 4 loci sequenced, i.e., hereafter refer to as 5

427 + 4); (2) taxa containing a minimum of three loci (i.e., 5 + 4 + 3); (3) taxa containing a minimum of

428 two loci (i.e., 5 + 4 + 3 + 2); and (4) all taxa (5 + 4 + 3 + 2 + 1). For each dataset, the appropriate

429 partition subsets were defined with PartitionFinder using the parameters described previously.

430 The subsets for the first and third datasets were the same seven as used for the supermatrix

431 containing all taxa (i.e., 5 + 4 + 3 + 2 + 1 dataset; see above). The 5 + 4 + 3 data set had an

432 additional subset for the third codon position of β-tubulin (Supplemental Table 5).

433   ML analyses for all four datasets were conducted with RAxML as described above.

434 Majority-rule consensus trees (70%) were built in Mesquite based on sets of 1,000 bootstrap

435 trees generated with RAxML for the four concatenated datasets. The Mesquite module Hypha

436 (Oliver et al., 2013; see also Miadlikowska et al., 2014) was used to integrate support values

437 derived from all applicable consensus trees onto each internode of the best tree derived from the

438 complete concatenated dataset (i.e., 5 + 4 + 3 + 2 + 1) (Fig. 1). No significant conflict was

439 detected among these trees except at the very tip of the H1 clade (Fig. 1).

440   Phylogenetic diversity (i.e., the sum of all the edge lengths in the subtree given by the tip

441   subset; PD) of Xylariaceae taxa was calculated in R (R Core Team) with the package caper

442   (Orme et al., 2013) and the topology generated from ML analysis of the 5 + 4 + 3 + 2 + 1

443   supermatrix. To assess whether newly collected isolates significantly increased the phylogenetic

444   diversity of the Xylariaceae, PD was calculated 1,000 times for subsets of 286 taxa (77 newly

445   collected OTU plus a random selection of 209 previously named Xylariaceae taxa), which is equal

446   to the total number of previously named Xylariaceae taxa in the tree. The observed PD of all

447   previously named Xylariaceae taxa was compared to this distribution to generate a distribution-

448   independent p-value (Supplemental Fig. 2). The R code is available at XXXX.

449

450   *2.8*. Comparison *of newly collected strains with known taxa*

451

452   To assess the potential novelty of newly cultured isolates when the topology of the tree alone is

453   inconclusive, we compared ITS rDNA sequences of taxa present in the Xylariaceae tree (when

454   available) to ITS rDNA sequence data for 205 Xylariaceae taxa identified to species but not

455   represented in the multi-locus dataset due to lack of sequences for non-ITS rDNA loci in NCBI

456   (Supplemental Table 6). Sequences were clustered into OTU at 95%, 97%, 99% and 100% using

457   ESPRIT and mothur following methods described above (see also U'Ren et al. 2012 for

458   methods). These data were used to assess the number of cases in which a newly cultured taxon

459   was found within the same 95% OTU as a named taxon (see Table 1; Fig. 2).

460

461   *2.9. Examining host breadth, substrate diversity, and geographic distribution of Xylariaceae*

462

463   We next identified taxa from previously published studies that are closely related to taxa in our

464   final data set. Most Xylariaceae are not represented by multiple loci in GenBank; instead, they are

465   represented (when present) by ITS rDNA sequences. We accessed this larger sampling to

466   address questions with regard to host breadth, substrate diversity, and geographic distribution

467   across the multi-locus phylogenetic framework developed here.

468   ITS rDNA sequences for newly cultured isolates from our continental surveys, as well as

469   reference taxa, were queried against NCBI's nr database using an e-value cutoff of $1 \times e^{-3}$. The

470   output was filtered to remove self hits, accessions from U'Ren et al. (2010; 2012), and hits with

471   percent identity <99%. If the same accession was a hit for multiple taxa, the hit with the highest

472   bit score was selected and the duplicate removed. A small fraction of BLASTn hits representing

473   sequences from uncultured isolates (i.e., clones or next-generation sequences) also were

474   excluded (n = 24; 1.9% of filtered hits).

475   From the list of filtered accession numbers, the geographic origin, host lineage (e.g.,

476   Angiosperm), and substrate information (e.g., surface sterilized leaf) for each isolate were

477   extracted from the respective metadata in GenBank and parsed using custom scripts (XXXX)

478   (Supplemental Tables 7-8). In the cases of missing metadata, information was gathered (when

479   possible) from manuscripts in which the sequences were published. Information on geographic

480   distribution, host, and substrate for reference taxa also was collected from published species

481   descriptions and online resources (Supplemental Table 9).

482   To visualize metadata in a phylogenetic framework, information for each terminal taxon

483   was classified into broadly defined categories (Fig. 2). Categories for provenance included (1)

484   U.S., Canada; (2) Mexico, South/Central America, Caribbean; (3) Europe, Russia; (4) Asia; and

485   (5) "Other" (e.g., Hawaii, Australia, New Zealand, Papua New Guinea, Africa, the Middle East,

486   and Antarctica, grouped as "other" due to a paucity of metadata from those sites). Isolates from

487   the Hawaiian Islands were included in "other" rather than U.S./Canada due to their geographical

488   isolation from the continental U.S. Categories for host breadth included (1) angiosperm; (2)

489   "gymnosperm" (i.e., Pinophyta and *Ginkgo biloba*); (3) spore-bearing vascular plant (i.e.,

490   lycophytes and ferns); (4) bryophyte (i.e., mosses and liverworts) and (5) lichen (LT). Substrate

491   categories included (1) living plant leaves or non-woody stems (LP); (2) dead plant leaves in

492   canopy (DP); (3) fallen plant leaves in leaf litter (FP); (4) wood/bark; (5) root; (6) seed; (7) soil; (8)

493   insect-associated; and (9) fallen fruits/inflorescences (Fig. 2). Data for each isolate were added to

494   the phylogenetic tree using a custom R script (XXXX).

495

496   *2.10. Macromorphological characterization of newly collected strains*

497

498   Newly collected isolates representing each putative species were subcultured from water

499   vouchers onto 2% MEA (20 g/L of malt extract [Amresco, Solon, OH, USA] and 15 g/L of agar

500   [Fisher Scientific, Pittsburgh, PA, USA]) and grown at room temperature under ambient light

501   conditions. To verify the identity of each isolate prior to macromorphological characterization,

502   DNA was extracted from each culture using the RedExtract-N-Amp Plant Kit (Sigma-Aldrich)

503   following a modified protocol. Under sterile conditions a small piece of mycelium was placed in a

504   1.5 ml tube with 100 µl of extraction buffer and 100 µl of 0.5 mm zirconium oxide beads (Next

505   Advance, Averill Park, NY, USA). After bead-beating for 1 min, the mycelium was incubated for

506   10 min at 95°C, after which 100 µl of dilution solution was added and the solution was vortexed

507   briefly. DNA was stored at -20°C until used for PCR. The ITS-partial LSU rDNA was amplified by

508   PCR with the primer pair ITS1F/LR3 using REDExtract-N-Amp PCR Ready Mix (Sigma-Aldrich,

509   St. Louis, MO, USA) following the manufacturer's recommendations and the PCR protocol

510   described in Arnold et al. (2007). PCR products were visualized and sequenced as described

511   above. Sequences were verified as 100% identical to expected sequences for each strain. After

512   verification, a 5 mm wide plug of each culture was transferred to 2% oatmeal agar (OA) plates to

513   induce sporulation (Ju et al., 2005). Cultures were grown under ambient light/dark condition at

514   room temperature (ca. 21.5°C) for up to six months before colony morphology was photographed

515   (Supplemental Fig. 3).

516

517   **3. Results and Discussion**

518

519   In addition to well-known saprotrophs, previous studies have revealed numerous endophytic

520   Xylariaceae in biomes ranging from high latitudes to the tropics (e.g., Arnold et al., 2009; Brunner

521   and Petrini, 1992; Carroll and Carroll, 1978; Davey et al., 2014; Davis et al., 2003; Del Olmo-Ruiz

522   and Arnold 2014; Higgins et al., 2007; Okane et al., 2012; Osono 2006; Petrini and Petrini, 1985;

523   Petrini et al., 1995). Although tropical Xylariaceae occurring in living and dead plants and lichens

524 are considered to be particularly diverse, our surveys captured many species of xylariaceous

525 fungi in subtropical, temperate, and boreal hosts (U'Ren, 2011; U'Ren et al., 2012). Incorporating

526 this large collection of plant- and lichen-associated strains from diverse sites in North America

527 into multi-locus phylogenetic analyses with previously described taxa significantly increases the

528 known phylogenetic diversity of Xylariaceae and provides an enriched perspective on

529 relationships proximate to, and within, this ecologically diverse family (Fig. 1, Fig. 2,

530 Supplemental Fig. 1, Supplemental Fig. 2).

531

532 *3.1. Phylogenetic delimitation of Xylariaceae within Xylariomycetidae*

533

534 Most taxa currently recognized as Xylariaceae formed a single clade in our single-locus

535 (phylogenies not shown) and multi-locus analyses (Supplemental Fig. 1). However, several taxa

536 previously described as Xylariaceae (*Anthostomella torosa* AFTOL-ID 732, *Dicyma funiculosa*,

537 and *D. pulvinata*; Ju et al., 1993; Kohlmeyer and Volkmann-Kohlmeyer, 2002; Peláez et al., 2008;

538 Stchigel and Guarro, 1998; Udagawa et al., 1994) appeared outside of this family in single-locus

539 and four-locus analyses (Supplemental Fig. 1). Athough placement of these taxa is uncertain due

540 to low support, both *Dicyma* and *Anthostomella* are known to be problematic with regard to

541 phylogenetic placement (Peláez et al., 2008; also see Daranagama et al., 2015).

542   Xylariaceae and Diatrypaceae have been proposed previously as sister families due to

543 shared morphological features (Parguey-Leduc, 1972; Schrantz, 1960; Rogers, 1979a). However,

544 a recent analysis suggests Xylariaceae may be more closely related to Cainiaceae (Senanayake

545 et al., 2015; see also Maharachchikumbura et al., 2015). In our phylogenetic analysis of the

546 subclass Xylariomycetidae the Cainaceae (represented here by *Seynesia erumpens*, was more

547 closely related to Xylariaceae than the Diatrypaceae (with moderate support, Supplemental Fig.

548 1). When members of Diatrypaceae were used as the outgroup in our analysis focusing on

549 Xylariaceae, *Seynesia erumpens* was nested within the ingroup but outside known Xylariaceae

550 (Fig. 1). *Seynesia* was classified previously in Amphisphaeriaceae (Eriksson and Hawksworth,

551 1991a; 1993), but was moved to Xylariaceae based on ascospore morphology (see Barr, 1990;

552   Eriksson and Hawksworth, 1991b; Hyde, 1995). A recent phylogenetic analysis based on ITS +

553   LSU rDNA placed the genus in Cainiaceae (Senanayake et al., 2015). Our analysis is not in

554   conflict with that placement of *Seynesia,* but uncertainty regarding its family-level placement

555   precludes confident delimitation of the Xylariaceae and family-level placement of the endophyte

556   clades E1 and E2 (Fig. 1, Fig. 2). In future work, multi-locus data for members of the Cainiaceae,

557   Lopadostomaceae, and Coniocessiaceae will be important to clarify the precise delimitation of

558   Xylariaceae.

559   More generally, the placement of many taxa within Xylariomycetidae was challenging due

560   to low support values at many deep internodes. This speaks to a general challenge in the

561   systematics and taxonomy of the subclass and its member families (e.g., Senanayake et al.,

562   2015), leading us to examine the contributions of the loci included in phylogenetic analyses. The

563   final Xylariomycetidae dataset consisted of four loci (ITS rDNA, LSU rDNA, *RPB2*, and β-tubulin

564   and contained a total of 4,700 characters (Supplemental Table 3). In agreement with previous

565   studies of other Ascomycota (e.g., Miadlikowska et al., 2014), alignments of protein coding genes

566   yielded a greater proportion of alignable nucleotides than did the ribosomal genes (Supplemental

567   Table 3). Although the alignment of LSU rDNA was slightly longer than that for *RPB2* (1,272 bp

568   vs. 999 bp, respectively), 97% of nucleotides were unambiguously aligned for *RPB2*, whereas

569   nearly half (46%) of nucleotides in the LSU rDNA alignment could not be aligned unequivocally

570   due largely to the presence of introns (Supplemental Table 3). For the β-tubulin alignment, only

571   exons could be aligned with confidence, but exons contain limited variation (618 distinct

572   alignment patterns). Thus, introns for β-tubulin were recoded using PICS-Ord (Lücking et al.,

573   2011), yielding an alignment with 1,664 distinct alignment patterns. No significant conflict was

574   observed between β-tubulin gene trees with and without the recoded characters, and the percent

575   of nodes with bootstrap support ≥70% increased from 38.8% to 60.9%. A similar strategy was

576   used to recode ITS1 rDNA and ITS2 rDNA, as these hypervariable regions could not be aligned

577   reliably across such distantly related taxa (see Bruns, 2001). Inclusion of recoded ITS rDNA

578   increased the number of alignment patterns for this region from 58 to 305, and the number of

579   well-supported nodes increased from 1.5% to 25.4% in the single locus tree. Accordingly,

580 additional sequences of phylogenetically informative loci from extended taxon sampling will be

581 necessary to clarify the ordinal- and family-level relationships within the Xylariomycetidae.

582

583 *3.2. Placement of several newly cultured isolates outside Xylariaceae illustrates conflicts with*

584 *BLASTn results*

585

586 Overall, 13 of 92 putative species from our surveys were tentatively identified as Xylariaceae

587 based on BLASTn hits for ITS-partial LSU rDNA, but were placed outside of the Xylariaceae in

588 our four-locus analysis (Table 1, Supplemental Fig. 1). These 13 OTU represent endophytes

589 (nine OTU) and saprotrophs (four OTU) and comprise 118 isolates overall (Table 1;

590 Supplemental Table 1). They were placed in clades containing species from four different families

591 of Xylariomycetidae or in clades containing no closely related reference taxa; however, their

592 phylogenetic placements are inconclusive due to low bootstrap support and insufficient taxon

593 sampling (Supplemental Fig. 1).

594 Discrepancies between BLASTn and phylogenetic analyses could occur because of (1) a

595 lack of closely related isolates in GenBank (especially problematic when analyses were restricted

596 only to named sequences), (2) high sequence similarity to species previously thought to be in the

597 Xylariaceae but of uncertain placement under current systematics frameworks (i.e., top BLASTn

598 hit to *Creosphaeria sassafras*; see also Supplemental Fig. 1), or (3) misidentified sequences in

599 GenBank (Peršoh et al., 2009; Vilgalys, 2003). Examination of BLAST results illustrates that

600 seven of the 13 OTU had top BLASTn hits to *Anthostomella conorum* CBS 119333 (EU552099).

601 The remaining five OTU had top BLASTn hits to unknown fungi with the closest named taxa in

602 the Xylariaceae. These results reiterate that assigning taxonomy (even at the family level) for

603 unknown isolates based solely on the top ITS BLASTn hit can be problematic (see also Stadler et

604 al., 2013; Gazis et al., 2012; U'Ren et al., 2009; but see Kõljalg et al., 2013 regarding the UNITE

605 ITS rDNA database).

606

607 *3.3. Subfamily structure within Xylariaceae*

608

609   We used five loci (ITS rDNA, LSU rDNA, *RPB2*, β-tubulin, and α-actin) to examine the

610   relationships of 298 taxa classified as Xylariaceae (>200 species) from 24 genera representing

611   two recognized subfamilies (Hypoxyloideae and Xylarioideae; Supplemental Table 1), in

612   conjunction with 79 potentially novel species representing 1,815 isolates of endophytic and

613   saprotrophic Xylariaceae from sites across North America (Table 1; Fig. 1, Fig. 2). Two putative

614   species from our surveys were removed from final analyses due to conflict among loci, resulting

615   in the inclusion of 77 putative species (OTU) from our collections.

616   Genera within the Xylariaceae generally are organized into the subfamilies based on

617   geniculosporium- or nodulisporium-like conidiophores in the anamorphic state (Ju and Rogers,

618   1996). However, the family currently contains numerous genera of uncertain placement due to

619   anamorphs that differ from those above (e.g., libertella-like conidiophores) or have unknown

620   conidial states (Ju and Rogers, 1996; Stadler et al., 2013). Previous analyses of the Xylariaceae

621   focused only on select taxa (e.g., Peršoh et al., 2009; Tang et al., 2009), were based on single

622   loci (e.g., ITS rDNA; Peláez et al., 2008; Sánchez-Ballesteros et al., 2000), or were restricted to

623   one subfamily (Hypoxyloideae, Hsieh et al., 2005; Xylarioideae, Hsieh et al., 2010), such that the

624   phylogenetic relationships of the two subfamilies and the placement of numerous genera *incertae*

625   *sedis* could not be addressed.

626   Although the clades containing currently recognized Hypoxyloideae and Xylarioideae

627   (sensu Stadler et al., 2013) were highly supported in our analyses, the two subfamilies were not

628   recovered here as sister clades (Fig. 1). Instead, our analyses suggest that the Xylariaceae may

629   be divided into three main lineages: (1) *Hypoxylon*, *Daldinia*, *Annulohypoxylon,* and closely

630   related genera; (2) a clade comprised of *Durotheca* and *Biscogniauxia* (*Biscogniauxia, Camillea*,

631   and *Obolarina*); and (3) Xylarioideae and related taxa. However, additional data is needed to

632   confirm these relationships with high support values. Our analysis places four endophyte-only

633   clades (E4-E7) as a grade preceding the origin of the Xylarioideae as currently delimited (see 3.5,

634   below; see also Fig. 1). These clades contained 12 of 77 putative species of Xylariaceae from our

635   collections (Fig. 1). Overall, early divergences of endophyte clades associated with each

636   subfamily and the Xylariaceae as a whole suggest an early origin of endophytic and/or

637   endolichenic fungi associated with diverse photosynthetic partners.

638

639   *3.4. Phylogenetic perspectives on Hypoxyloideae*

640

641   Overall, 28 of 77 putative species from our collection (i.e., 26 endophytic and two saprotrophic

642   OTU) were placed within the Hypoxyloideae in our multi-locus analyses, or in affiliation with

643   *Whalleya*, here treated as within Hypoxyloideae (see below). These strains were placed in all

644   genera of Xylariaceae included in the analysis with the exception of *Durotheca* and *Obolarina*.

645   Results of our analyses are largely congruent with the study of Hypoxyloideae by Ju et al.

646   (2007). However, in our topology *Whalleya microplaca* was placed in a clade outside of species

647   currently classified as Hypoxyloideae, whereas Ju et al. (2007) suggested a close relationship of

648   *Whalleya* with species of *Biscogniauxia* and *Theissenia* (renamed *Durotheca*; see Læssøe et al.,

649   2013). Developmentally, *Whalleya microplaca* resembles *Biscogniauxia* (i.e., the presence of

650   bipartite stromata), but stromata and anamorphs are morphologically similar to diatrypaceous

651   fungi (e.g., anamorphic isolates of *Whalleya* have libertella-like conidiogeneous structures;

652   Rogers et al., 1997b; Stadler et al., 2014; see also Glawe and Rogers, 1986). Our analyses

653   placed three endophyte OTU with *W. microplaca* (clade E3, Fig. 1) and placed E3 as sister to the

654   known Hypoxyloideae, with strong support from two bootstrap analyses. Pending morphological

655   examination, we suggest that the Hypoxyloideae may be expanded to include clade E3 (Fig. 1).

656   Within Hypoxyloideae, Hsieh et al. (2005) recognized three major clades based on ML

657   and Bayesian analyses of β-tubulin and α-actin genes: the *Biscogniauxia* clade, the

658   *Annulohypoxylon* clade, and the *Hypoxylon*/*Daldinia* clade. Our analyses recovered a well-

659   supported clade containing all of the *Hypoxylon, Daldinia*, and *Annulohypoxylon* taxa, as well as

660   an isolate of *Rostrohypoxylon terebratum* (see Fournier et al., 2010 for a description of the genus

661   and its paraphyly) (Fig. 1). Within this clade, *Daldinia* and *Annulohypoxylon* form well supported

662   clades in our analyses (Fig. 1). However, species of *Hypoxylon* were found in multiple clades and

663 relationships among the previously defined subclades H1, H2, and H3 (sensu Hsieh et al., 2005)

664 were not confirmed (Fig. 1).

665   Additionally, the position of *Biscogniauxia* as sister to the clade containing *Hypoxylon*,

666 *Annulohypoxylon*, and *Daldinia* (see Hsieh et al., 2005; see also Læssøe et al., 2013) was not

667 recovered here. Our topology suggests that *Biscogniauxia* and *Durotheca* (see Læssøe et al.,

668 2013 for genus description) are sister to one another, but the relationship is not well supported

669 and the placement of this two-genus clade relative to other genera in the Hypoxyloideae is

670 uncertain in our analysis. A sister relationship between these genera would agree with results of

671 high performance liquid chromatography (HPLC) illustrating that the chemical profiles of

672 *Biscogniauxia* and *Durotheca* are closer to each other than to *Hypoxylon* and *Daldinia* (Læssøe

673 et al., 2013). In turn, the highly supported *Biscogniauxia* clade contained isolates of *Camillea*

674 *tinctor* and *Obolarina dryophila* (see Pažoutová et al., 2010b for discussion of *Biscogniauxia*

675 paraphyly).

676

677 3.5. *Phylogenetic perspectives on Xylarioideae*

678

679 Overall, 34 of 77 putative species from our collection (i.e., 32 endophytic and two saprotrophic

680 OTU) were placed within the Xylarioideae as currently circumscribed. Although endophyte OTU

681 were found within all major lineages of the subfamily, they were most abundant in the "NR" and

682 "HY" clades (sensu Hsieh et al., 2010; Figs. 1, and 2D).

683   Hsieh et al. (2010) grouped members of the Xylarioideae into four major clades: (1) the

684 clade containing *Xylaria* spp. associated with termite nests (i.e., subgenus *Pseudoxylaria*; TE); (2)

685 the clade containing *Xylaria hypoxylon* and closely related species (HY); (3) the clade containing

686 *Nemania*, *Rosellinia*, *Entoleuca*, and *Euepixylon* (NR); and (4) the clade containing *Xylaria*

687 *polymorpha* and closely related species (PO). Our analyses strongly support the monophyly of

688 these clades and relationships among them, with the exception of the node encompassing all four

689 clades, which was recovered in all four analyses but never with support values ≥ 70% (Fig. 1).

690 Overall, relationships among terminal branches were highly similar to those reported for

691 described species, and our analyses confirm the early divergence of the *Poronia-Podosordaria*

692 clade within the Xylarioideae (see Hsieh et al., 2010).

693   Our results further suggest a potential expansion of the current circumscription of

694 Xylarioideae. Clades E4-E7, which subtend the currently recognized subfamily, include 12

695 endophyte OTU as well as an *Anthostomella* sp. from Puerto Rico (Fig. 1, Fig. 2C). The

696 phylogenetic placement of two of these three lineages is well supported in our analyses. Two

697 OTU have highly similar ITS rDNA sequences to *Anthostomella* spp. that were not represented in

698 the tree due to lack of multi-locus data (NC1622 with *Anthostomella sepelibilis* strain F-160, 797;

699 AZ1047 with *Anthostomella pinea* CBS 128205) (Table 1, Fig. 2C). However, FL1105, which is

700 placed among isolates with hits to *Anthostomella*, has >95% ITS rDNA sequence identity to

701 *Muscodor vitigenus* in NCBI (Table 1, Fig. 2C). The genus *Muscodor* initially was proposed to

702 include an endophytic fungus that produces antimicrobial volatiles (Worapong et al., 2001), but its

703 taxonomic placement in the Xylariaceae was uncertain (see Stadler et al., 2013). Placement of

704 FL1105 in clade E5 and the monophyly of this clade is highly supported in our Xylariaceae tree

705 (Figs. 1, 2C). Overall, our results suggest that *Anthostomella* is polyphyletic based on the

706 positions of AK1471, FL1651, AZ1047, and NC1622, which are placed in different clades despite

707 high sequence similararity to previously described species of *Anthostomella* (Table 1; Fig. 1; also

708 see Lu et al., 2000; Daranagama et al., 2015). Based on the strong phylogenetic support reported

709 here (Fig. 1, Fig. 2C) and pending morphological examination, we suggest that the Xylarioideae

710 may be expanded to include clades E5-E7.

711

712 *3.6. Limitations of current data sets*

713

714 As noted in previous studies, ribosomal genes alone do not provide enough phylogenetic

715 information to confidently resolve infrafamilial relationships in Xylariomycetidae (Duong et al.,

716 2004) or the evolutionary history among genera of Xylariaceae (Peláez et al., 2008; Tang et al.,

717 2009). In these circumstances, protein-coding genes such as *RPB2* can provide additional,

718 phylogenetic signal that result in more resolved phylogenies of Xylariaceae with higher statistical

719 support for clades (Tang et al., 2007; see also Reeb et al., 2004). Other protein-coding genes,

720 such as β-tubulin and α-actin, also have been used to reconstruct evolutionary relationships for

721 *Hypoxylon* and closely related genera of Xylariaceae (Hsieh et al., 2005). However, in the present

722 study we found that the protein coding genes β-tubulin and *RPB2* were insufficient to resolve

723 infrafamilial relationships in the Xylariomycetidae even when combined with ribosomal loci (ITS

724 rDNA, 5.8S rDNA, LSU rDNA). Analyses of the Xylariaceae using five loci (β-tubulin, α-actin,

725 *RPB2*, ITS plus 5.8S rDNA, and LSU rDNA) also failed to provide high statistical support for many

726 clades within the family.

727 ITS1 and ITS2 rDNA and introns of β-tubulin and α-actin were too divergent to be aligned

728 unambiguously at the ordinal or familial level. However, we used a non-alignment based method

729 (PICS-ord; Lücking et al., 2011) to extract phylogenetic information from these regions that were

730 excluded from the alignments subjected to phylogenetic searches. This method recovered a

731 substantial amount phylogenetic signal and greatly reduced phylogenetic uncertainty.

732 Overall, our results indicate that resolving evolutionary relationships in the Xylariaceae

733 and Xylariomycetidae with high confidence will require sequence data from additional protein-

734 coding genes that can be aligned unambiguously at broad taxonomic levels. Data from the gene

735 for RNA polymerase II largest subunit (*RPB1*), the gene for the minichromosome maintenance

736 complex component 7 (*MCM7*) (Chen et al., 2015; Miadlikowska et al., 2014), and/or additional

737 new molecular markers derived from the AFToL 2 project (aftol.org) are likely to be useful. Our

738 results also argue for increased taxon sampling of both endophytes and morphologically delimited

739 species not currently represented in public sequence databases.

740

741 *3.7. Phylogenetic perspectives on the potential novelty of newly collected strains*

742

743 Putatively novel species of Xylariaceae isolated from plants and lichens across North America

744 were frequently placed in previously described, major clades of Xylariaceae in both subfamilies,

745 including clades containing well-known plant pathogens (i.e., *Biscogniauxia*, *Kretzschmaria*),

746 saprotrophs (i.e., *Xylaria*), and clades thought to be specific to insects or animal dung (i.e.,

747   *Pseudoxylaria* clade and *Poronia + Podosordaria* clade, respectively; see Krug et al., 2004; Hsieh

748   et al., 2010; Rogers et al., 2005; Visser et al., 2009; Figs. 1 and 2C).

749   Although we recovered endophyte-only clades distinct from known or previously

750   sequenced isolates (e.g., E1, 2, 4, 5, 7, 8, and 9), each of which included one ore more putative

751   species, numerous OTU were resolved as closely related to previously described taxa. For

752   example, nine endophyte and saprotroph OTU were placed within a well-supported monophyletic

753   clade with multiple representatives of representative species (Fig. 1, Fig. 2; FL1408 within the

754   *Daldinia eschscholzii* clade; AK1016, AZ0526, AK0128, AK0995 within the *D. loculata* clade;

755   FL1170 within the *Hypoxylon rubiginosum* clade; AZ0703 within the *Biscogniauxia mediterranea*

756   clade; AK0226 within the *Nemania serpens* clade; and NC1011 within the *Xylaria cubensis*

757   clade).

758   However, in cases where endophytic fungi are sister to a single representative of a

759   described species, the topology of the tree alone does not help us determine whether unknown

760   isolates represent the asexual or anamorphic life stage of a described teleomorphic species or a

761   potentially novel closely related species. To address this uncertainty we analyzed the similarity of

762   our isolates to described species in the tree using ITS rDNA clustering. Despite the fact that

763   undescribed isolates are often found in the same clade or are sister to described species, only 14

764   of 77 Xylariaceae OTU isolated in our surveys (i.e., 18.2%) were part of the same 95% OTU as a

765   described species present in the tree (Fig. 2).

766   We further analyzed the ITS rDNA similarity of endophytic and saprotrophic fungi with

767   205 named Xylariaceae taxa (n = 85 species) represented in GenBank by ITS rDNA sequences,

768   but not by β-tubulin, α-actin, *RPB2*, or LSU rDNA (and thus not included in the phylogenetic

769   analyses; Supplemental Table 6). This information, presented within a phylogenetic framework,

770   accesses a large pool of publicly available sequences that exceeds the taxonomic representation

771   available based on other loci or multiple loci. Based on ITS rDNA OTU clustering at 95%

772   similarity, 10 additional OTU found in the multi-locus analysis are highly similar to named

773   sequences in NCBI (see Table 1). Two additional OTU also are similar to sequences in NCBI

774   based on ITS rDNA clustering, but were removed from the concatenated supermatrix due to

775   conflict (Table 1). Combining ITS rDNA similarity in a phylogenetic framework identified several

776   GenBank sequences with taxonomic names in apparent conflict with tree topology (e.g., *Xylaria*

777   *mellissii* F-048,697 found within *Nemania* + *Rosellinia* clade, Fig. 2D; also see above for

778   discussion of *Anthostomella* spp). Only five of the 12 taxa whose ITS rDNA sequences match our

779   isolates are vouchered in easily accessed culture collections (e.g., Centraalbureau voor

780   Schimmelcultures [CBS], American Type Culture Collection [ATTC], Agricultural Research

781   Service Culture Collection [NRRL]) (Table 1; Supplemental Table 6), thus precluding additional

782   morphological and molecular characterization.

783   Overall, 44 of 79 (55.7%) xylariaceous species collected in our surveys (representing 42

784   endophyte and two saprotroph OTU) appear to lack closely related, described species (Table 1;

785   Fig. 2). Of these apparently novel OTU, 34 also lacked closely related BLASTn hits (≥99% ID) to

786   other unnamed fungi in GenBank (Fig. 2). Importantly, these results are based only on the

787   species diversity represented in public databases, and thus do not assess novelty with respect to

788   those species of Xylariaceae known only from their teleomorphs or from drawings, or otherwise

789   not represented in public databases (discussed by Stadler et al., 2013).

790   Such conclusions are dependent on a defined level of ITS rDNA sequence similarity;

791   however, the degree of intraspecific ITS rDNA variability differs among taxonomic groups (see

792   Nilsson et al., 2008). For five *Xylaria* spp., U'Ren et al. (2009) reported low intraspecific variation

793   in ITS rDNA (1.43% ± 2.94%), and variation between sister taxa averaged 4.18% ± 2.18%.

794   However, Stadler et al. (2013) noted that different morphological species can have identical ITS

795   rDNA sequences (e.g., *Daldinia concentrica* and *D. steglichii*). Across both subfamilies, we found

796   12 cases where different morphological species shared the same 95% OTU designation (e.g.,

797   *Xylaria plebeja*, *X. luteostromata* var. *macrospora*, and *X. intracolorata* designated OTU 265; Fig.

798   2). This appears more common for species of Xylarioideae than Hypoxyloideae (Fig. 2).

799   Accordingly, endophyte isolates putatively identified as previously described species may in fact

800   represent novel species. Indeed, we found cases where a comparison of the culture morphology

801   on oatmeal agar for described species (when available) differed from our observations for

802   endophytic isolates. For example, *Hypoxylon submonticulosum* (Ju and Rogers, 1996) is

803   described as a fast-growing isolate with pale-mouse grey, cinnamon, to grayish sepia color, with

804   sporulating regions scattered over the colony surface. However, an endophyte representing the

805   same OTU (NC0708) remained as sterile, white mycelia after six months in culture on the same

806   media (Supplemental Fig. 3).

807   Conversely, we found seven cases where isolates of the same putative species occurred

808   in different 95% OTU (*Xylaria cubensis*, *X. curta*, *Hypoxylon dieckmannii*, *H. crocopeplum*, *H.*

809   *fendleri*, *H. haematostroma*, and *Nemania serpens*). Assuming these isolates are correctly

810   identified and represent a single species rather than a species complex (e.g., as for *Fusarium*

811   *solani*; O'Donnell, 2000), we may overestimate the novelty of unknown fungi. For example, ITS-

812   partial LSU rDNA clustering of isolates collected in our surveys identified multiple endophyte OTU

813   at 95% sequence similarity (i.e., AK0995, AK0128, AZ0526, AK0222, and AK1016). However,

814   representatives of these isolates were placed within a well-supported clade containing two

815   isolates of *Daldinia loculata,* suggesting that these OTU are all *D. loculata* rather than multiple

816   species (Fig. 2). Interestingly, these isolates represent different macromorphologies on 2% MEA

817   and OA and will need additional morphological characterization (Supplemental Fig. 3). Thus,

818   incorporating representative isolates into a multi-locus phylogenetic framework is only the first

819   step in assessing the potential novelty of endophytic and saprotrophic fungi.

820

821   *3.8. Metadata availability, host breadth, substrate diversity, and geographic distribution of*

822   *Xylariaceae*

823

824   Many Xylariaceae species have broad distributions in forests across both the Northern and

825   Southern Hemispheres (e.g., *Daldinia* spp. Stadler et al., 2014), whereas other species appear to

826   be restricted geographically (e.g., *Xylotumulus gibbisporus* endemic to Hawai'i; Rogers and Ju,

827   2012). However, distribution data based on ascomata are likely to be incomplete due to the fact

828   that Xylariaceae species typically only form ascomata on a "preferred" host (e.g., *Obolarina*

829   *dryophila* on *Quercus*; Pažoutová et al., 2010b), despite the potential to live asymptomatically on

830   a wide diversity of plant species and substrates (e.g., Petrini and Petrini, 1985). Additionally,

831   species of Xylariaceae reported to have cosmopolitan distributions may potentially represent

832   complexes of cryptic species revealed only by detailed morphological, chemical, or molecular

833   analyses (e.g., *Daldinia eschscholzii*; see Stadler et al., 2004). Therefore, incorporating publicly

834   available sequence data (many representing cryptic microfungi, including endophytes) coupled

835   with records of ascomata can provide further information to estimate the geographic distribution,

836   host breadth, and substrate diversity of xylariaceous species.

837   Our sampling of endophytes from diverse plant and lichen species in five sites across

838   North America emphasizes that lichen thalli are a very common habitat for Xylariaceae (see also

839   Arnold et al., 2009). The interiors of apparently healthy lichen thalli yielded 1,259 isolates

840   representing 67 putative species (84.8% of the 79 xylariaceous OTU from our surveys considered

841   here; see Table 1). These fungi were isolated from 46 lichen species representing 10 major

842   lineages of mycobionts (7 orders and 3 families *incertae sedis* in Lecanoromycetes)

843   (Supplemental Table 1). Although the highly diverse nature of xylariaceous endophytes from

844   angiosperms has been previously reported, especially in tropical forests (Bayman et al., 1998;

845   Govinda Rajulu et al., 2013; Linnakoski et al., 2012; Okane et al., 2008; Whalley, 1996), our data

846   illustrate that in temperate and boreal communities, Xylariaceae are especially diverse in conifers,

847   lycophytes, and bryophytes (Supplemental Table 2; see also Davis et al., 2003).

848   Although these hosts harbored a high diversity of endophytes, we observed no clear

849   phylogenetic pattern with regard to host phylogeny (Fig. 2). The vast majority of endophytic

850   Xylariaceae appear to be host generalists: 91 of 124 terminal taxa (73.4%) capable of living

851   endophytically (i.e., reported at least once from living leaves, lichen thalli, or asymptomatic inner

852   bark, cortex, sapwood, or branches of a living host) occurred on more than one host lineage (e.g.,

853   angiosperm, "gymnosperm", spore-bearing vascular plant, bryophyte, or lichen; Fig. 2). There

854   was no apparent clade or species-level specificity with regard to lichen photobiont or mycobiont

855   (e.g., OTU found in cyanolichens also occurred in lichens with green algal symbionts;

856   Supplemental Table 1). At the community level, previous work suggested a unique connection

857   among fungi occurring in lichens and bryophytes (U'Ren et al., 2010; 2012), but 77.8% of 27 OTU

858   cultured in our surveys from both lichens and bryophytes also occurred in living tissues of

859 vascular plant hosts. Thus, non-xylariaceous taxa seem to account for the patterns observed by

860 U'Ren et al. (2010; 2012).

861   The majority of terminal taxa in the tree with an endophytic life stage also were recovered

862 from sencescent leaves or decomposing leaves, wood, bark, fruits, or flowers (74.2%; n = 92 of

863 124 terminal taxa; Fig. 2), suggesting that for many species endophytism is only one stage of a

864 complex lifecycle that can involve interactions with diverse host lineages. For example, early-

865 diverging endophyte OTU (i.e., FL0915, FL2044, and FL0641) were cultured from living tissues of

866 both lichens and angiosperms, as well as dead leaves of a conifer host (Table 1; Fig. 2). In

867 contrast, only 33 of 241 (13.7%) terminal taxa not found as endophytes were collected from

868 multiple host lineages and substrates, a pattern that may reflect greater ecological specialization.

869 Indeed, a few more recently diverged clades appear to have evolved more specific host ranges

870 (e.g., *Xylaria hypoxylon* aggregate on angiosperms; see also Læssøe and Lodge, 1994; Rogers,

871 1979b; Whalley, 1985). However, additional sampling of endophytes of tropical lichens and non-

872 angiosperm lineages is needed to confirm the pattern. Additionally, inferences can be limited due

873 to missing or incomplete metadata in public databases. For example, 32.8% and 46.7% of 1,264

874 filtered BLASTn hits lacked information for host lineage and substrate, respectively

875 (Supplemental Tables 7-8), revealing a pressing need for standardized formats and requirements

876 for metadata submission with sequences.

877   After incorporating both morphological records and GenBank metadata, we found that

878 155 of 365 terminal taxa (42.5%) were reported only in a single geographic region. Importantly,

879 geographic regions were broadly defined, such that a taxon reported from a single region might

880 still be geographically widespread within a region (e.g., *Xylaria tuberoides* in southern Mexico,

881 Venezuela, French Guiana, and Guyana). Over a third of terminal taxa (n = 137; 37.5%) were

882 found in ≥3 regions (e.g., US/Canada, S. Mexico/C. and S. America/Caribbean, Europe, Asia, or

883 "Other"; Fig. 2). When analyzed in a phylogenetic context, taxa with widespread geographic

884 ranges were distributed throughout the Xylariaceae. However, *Obolarina*, *Durotheca*,

885 *Pseudoxylaria*, *Podosordaria* and *Poronia*, and endophyte-only clades appear more

886 geographically limited (Fig. 2). The apparent localization of certain taxa or clades may be due to

887   the limited range of their host (e.g., *Pseudoxylaria* on Macrotermitinae termite nests; see above),

888   dispersal limitation, climate, or habitat restrictions (see Whalley, 1985), and/or missing metadata

889   (Supplemental Tables 6-7). Additional sampling from diverse locations and hosts, using both

890   culturing and culture-free next generation sequencing, will help clarify the biogeographic patterns

891   of xylariaceous fungi.

892

893   *3.9. Contributions of endophytes to understanding the ecology of described species:* Daldinia

894   loculata

895

896   Our analyses revealed a monophyletic clade containing five endophytic OTU interspersed with

897   two isolates of *Daldinia loculata* (Fig. 2B). Ascomata of *D. loculata* usually are recovered from

898   burnt or damaged Betulaceae in temperate, boreal, and montane forests of the Northern

899   Hemisphere, although the species also has been observed on wood of Salicaceae, Fagaceae,

900   and Rosaceae (Stadler et al., 2014). It has been reported previously as a foliar endophyte of non-

901   angiosperm hosts (see Pažoutová et al., 2010a). In our surveys, members of the *D. loculata*

902   clade were frequently isolated as endophytes of diverse plants and lichens in boreal and

903   subarctic Alaska. For example, in Eagle Summit, Alaska, we isolated *D. loculata* from

904   asymptomatic, living photosynthetic tissue of evergreen angiosperms, conifers, lycophytes, and

905   bryophytes and long-lived thalli of nine lichen species representing different growth forms,

906   substrates, and photobionts. ITS rDNA analyses suggest that closely related isolates occur in

907   Nome, Alaska, as well as Arizona, Florida, Jamaica, Europe, and New Zealand in a range of

908   biomes from subarctic tundra to tropical high-elevation forest (Fig. 2; Supplemental Tables 1, 6-

909   7). Although previously thought to be rare or absent from subtropical and tropical forests, these

910   data illustrate that the species has a wider geographic and host range than reported previously.

911   Additionally, our data may help illuminate aspects of the lifecycle of *D. loculata*.

912   Previously, the species was proposed to inhabit asymptomatic, living wood of *Betula* until fire kills

913   the host (Guidot et al., 2003; Johannesson et al., 2001a; 2001b; see also Rayner and Boddy,

914   1988). The fungus then grows rapidly within host tissue, forming conidia beneath the bark that are

915   dispersed by insects among burnt trees. When conidia of different mating types interact, the

916   sexual cycle is initiated, producing ascospores that are wind-dispersed to infect the unburned

917   wood of young saplings, where the fungus presumably is endophytic in woody tissues of the host

918   until another fire begins the cycle again (Guidot et al., 2003). Our data illustrate that *D. loculata*

919   was abundant in the photosynthetic tissues of living lichens and evergreen plants in Eagle

920   Summit (Alaska), as well as present in lower abundances in senescent leaves still attached in the

921   canopy and fallen leaves in leaf litter from the previous year) (i.e., DL, FP; Table 1; Supplemental

922   Table 1). However, we did not detect *D. loculata* in newly flushed leaves of *Salix* and *Betula*,

923   which suggests that leaves of these hosts may be colonized from airborne inoculum each

924   season. Whether inoculum is from wind-dispersed ascospores or asexual conidia produced on

925   senescent evergreen plants or lichens in the same site remains to be elucidated, but it implies

926   that the fungus is reproducing during intervals between fires (ca. 50-150 years in this area;

927   Johannesson et al., 2000).

928

929   *3.10. Contributions of endophytes to understanding the ecology of described species:* Xylaria

930   cubensis

931

932   Ascomata of *Xylaria cubensis* are commonly encountered on decomposing angiosperm wood in

933   tropical, subtropical, and temperate forests across the globe, degrading both lignin and cellulose

934   thereby causing a physiological white rot (Rogers, 1984). However, closely related isolates are

935   frequently cultured in endophyte surveys, especially of tropical angiosperms, ferns, and

936   lycophytes (e.g., Fan et al., 2014; Fröhlich et al, 2000; Okane et al., 2008; 2012; Rodrigues,

937   1994; Rodrigues et al., 1995; Rodrigues and Samuels, 1990; 1999; Rodrigues and Petrini, 1997)

938   (Fig. 2E). Our results indicate that *X. cubensis* (represented by NC1011) is a frequent inhabitant

939   of temperate and subtropical lichens (Fig. 2E; Table 1; Supplemental Table 1). Of the 193 *X.*

940   *cubensis* isolates in a single 95% ITS rDNA OTU, 154 were cultured from lichens, whereas the

941   remaining were found from angiosperms, conifers, and bryophytes (Supplemental Table 1; Fig.

942   2E). Even at a finer scale there was no evidence for host specificity: numerous unique ITS rDNA

943 genotypes (based on 100% sequence similarity) were shared among different host species,

944 substrates (lichens, living, and dead plant tissues), as well as geographic locations (see also

945 Okane et al., 2012; Supplemental Table 2). The species was collected from 22 species of lichens

946 in Arizona, North Carolina, and Florida, representing five mycobiont orders (Lecanorales,

947 Teloschistales, Ostropales, Peltigerales, and Umbilicariales) and diverse substrates, growth

948 forms, and photobionts (e.g., various Trebouxiales and *Nostoc* species) (Supplemental Table 1).

949 All investigated lichen genera in North Carolina and Florida (>10 spp. per site) yielded *X.*

950 *cubensis* with the exception of the epiphytic crustose lichen *Herpothallon rubrocintum*, the only

951 representative from the class Arthoniomycetes. However, isolates of *X. cubensis* are reported to

952 have high morphological, genetic, and chemical diversity (Casella et al., 2013; Rodrigues et al.,

953 1993; Rodriguez et al., 1995; also see above for discussion of intraspecific ITS rDNA variation)

954 and additional studies are necessary to elucidate whether *X. cubensis*, as currently defined,

955 represents a complex of several species, each with potentially different host and substrate

956 preferences.

957

958 *3.11. Conclusions*

959

960 The goal of this study was to address the impact of a large collection of plant- and lichen-

961 associated strains on the circumscription and phylogenetic structure of the Xylariaceae, and to

962 evaluate their evolutionary history and ecology in a multi-locus phylogenetic context. Our results,

963 coupled with previous molecular phylogenetic studies, reiterate the need for taxonomic revision at

964 several levels within the Xylariaceae (Hsieh et al., 2005; 2010; Pažoutová et al., 2010b). Such

965 revisionary work is hindered by the sheer diversity of xylariaceous fungi, a shortage of trained

966 mycologists, the lack of reproductive structures in culture for many strains, biases in existing data,

967 the lack of molecular data from type specimens (especially non-ITS rDNA sequence data) and

968 the need for additional phylogenetic molecular markers (see Stadler et al., 2013; Stadler et al.,

969 2014 for discussion of revision; also see Daranagama et al., 2015; Senanayake et al., 2015).

970 Given the current situation, it is premature to assign taxonomic names to the majority of our

971　endophytic and saprotrophic OTU. Importantly, representative cultures have been deposited in

972　the Gilbertson Mycological Herbarium (ARIZ), where they are available on request for additional

973　characterization. Additionally, this work detects potentially misidentified specimens and

974　sequences in public databases, illuminates large gaps in available metadata for sequences

975　deposited in NCBI, and identifies the need for novel methods to integrate ITS rDNA sequences

976　into robust, multi-locus phylogenetic analyses.

977　　　　More generally, our study expands current knowledge regarding the ecology, host use,

978　and geographic distributions of well-known Xylariaceae. We found that the majority of

979　xylariaceous endophytes obtained in large surveys in North America can be classified in the

980　hypoxyloid and xylaroid subfamilies, although numerous endophytes also were found outside of

981　these lineages (as currently circumscribed). Most newly cultured strains appear to represent

982　novel species rather than previously described species, but inferences are limited by the potential

983　for previously known Xylariaceae to be absent from public databases. Representatives of the

984　lineages associated with the origin of Xylariaceae were found in living, asymptomatic leaves of

985　angiosperms, gymnosperms, and bryophytes, consistent with the purported origin of endophytism

986　early in the evolution of the Pezizomycotina (Lutzoni et al., in review). Our data suggest that in

987　general, temperate and boreal xylariaceous endophytes have both endophytic (in both plants and

988　lichens) and saprotrophic life stages (i.e., many endophyte OTU also contained isolates found

989　inside non-living leaves (consistent with observations by Osono, 2002; 2005; 2006). However,

990　additional work is necessary to determine the saprotrophic capabilities of endophytic OTU

991　presented here, and the genomic, transcriptomic, and metabolic base of endophytic,

992　saprotrophic, and pathogenic modes in this compelling and diverse family.

993

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1667

1668 **Figure Legends**

1669

1670 **Figure 1.** Phylogenetic relationships among 365 Xylariaceae representatives, including 77 newly

1671 collected endophyte and saprotroph putative species (representing 78 isolates) from field surveys

1672 of plants and lichens in five sites across North America and 287 previously named taxa. The tree

1673 was inferred by maximum likelihood based on a combined five-locus dataset (i.e., ITS rDNA, LSU

1674 rDNA, *RPB2*, α-actin, and β-tubulin sequences, described in the text as the 5 + 4 + 3 + 2 + 1

1675 dataset). Two species of Diatrypaceae (*Diatrype disciformis* and *Eutypa lata*) were used to root

1676 the tree based on the results of a four-locus supermatrix analysis of the Xylariomycetidae

1677 (Supplemental Fig. 1) and the availability of multi-locus sequence data (Supplemental Table

1678 2). The four-box grid at each internode indicates maximum likelihood bootstrap support values

1679 derived from our cumulative supermatrix approach (see legend and Materials and Methods).

1680 Major clades of Hypoxyloideae (*Hypoxylon* clades "H1", "H2", and "H3", *Daldinia* clade,

1681 *Annulohypoxylon* clade, *Durotheca*, and *Biscogniauxia* clade) are defined according to Hsieh et

1682 al. (2005) and Læssøe et al. (2013). Major clades of Xylarioideae are defined according to Hsieh

1683 et al. (2010): subgenus *Pseudoxylaria* (*Pseudoxylaria* "TE" clade); *Xylaria hypoxylon* and closely

1684 related taxa (*Xylaria* "HY" clade); the clade containing *Nemania* spp.*, Rosellinia* spp. and

1685 *Entoleuca mammata* (*Nemania* + *Rosellinia* "NR" clade); and the clade containing *Xylaria*

1686 *polymorpha* and closely related taxa (*Xylaria* "PO" clade). Red and dark-blue fonts indicate taxa

1687   included in analyses by Hsieh et al. (2005) and Hsieh et al. (2010), respectively. Black font

1688   indicates isolates from studies listed in Supplemental Table 2 (with the exception of *Obolarina*

1689   and *Durotheca*, none of the genera listed in black has been described formally using both

1690   morphological and molecular data). Light blue strain- and species names indicate specimens

1691   sequenced for the present study. Representative isolates from our surveys are shown as a two-

1692   letter code (AZ, NC, FL, AK) followed by the isolate number, the host and substrate information

1693   for the 95% ITSrDNA-partial LSU OTU represented by that isolate (LT, lichen thallus; LP, living

1694   plant tissue; DP, dead leaves in the canopy; and FP, dead plant leaves in leaf litter), and the total

1695   number of isolates in the OTU (Table 1). Subfamily classifications (i.e., Hypoxyloideae or

1696   Xylarioideae) are based on Stadler et al. (2013). Genera currently unclassified at the subfamily

1697   level (i.e., *Whalleya* and *Anthostomella*; see Stadler et al., 2013), as well as endophyte clades

1698   E3-E7 are indicated on the tree as incertae subfamiliae. Two endophyte OTU (clades E1-E2) as

1699   well as *Seynesia erumpens* are labeled as incertae familiae reflecting the recent proposal of

1700   *Seynesia* as a member of the Cainaceae rather than Xylariaceae (see Senanayake et al., 2015).

1701   The blue star denotes *Hypoxylon argillaceum* CBS 527.63, a potentially misidentified isolate for

1702   which taxonomic revision may be warranted.

1703

1704   **Figure 2.** Phylogenetic integration of geographic and ecological metadata in the evolutionary

1705   context of the Xylariaceae (Fig. 1). Tree topology and clade definitions follow Fig. 1. Thickened

1706   branches indicate maximum likelihood bootstrap values ≥70% from all four bootstrap analyses of

1707   the cumulative supermatrix approach. An asterisk (*) denotes nodes with ≥70% support from at

1708   least one of the bootstrap analyses from the cumulative supermatrix approach (e.g., 5 + 4) (see

1709   Fig. 1). Major clades are shaded according to the ecological mode of the majority of taxa used in

1710   the multilocus analysis: green shading, endophyte clade (numbered E1-E9); grey, saprotroph;

1711   white, pathogen; brown, dung-associated; peach, termite-associated; and purple, equivocal.

1712   Within clades, terminals are labeled with colored fonts to indicate ecological mode of the terminal

1713   taxon based on review of the literature (see Supplemental Table 9 and legend). Within taxon

1714   names, numbers after the dash indicate the 95% OTU designation based on clustering analysis

1715   of ITS rDNA sequences for all isolates (when available) included in the phylogenetic analyses

1716   (e.g., *Daldinia vernicosa* 121—139 belongs to OTU 139). In cases where an isolate recovered in

1717   our surveys was within the same 95% ITS rDNA group as a named taxon not present in the tree

1718   due to lack of multi-locus data, the species name follows the OTU designation (e.g., FL1857 − 83

1719   − *Hypoxylon pulicicidum*; see also Table 1). Columns of numbers after taxon names indicate (1)

1720   the total number of isolates recovered in our surveys represented per 95% ITS rDNA group (see

1721   also Table 1) and (2) the number of BLASTn hits with ≥99% identity (isolates lacking numbers did

1722   not have ITS rDNA sequences in NCBI) (Supplemental Tables 7-8). The geographic locations of

1723   collection, photobiont host lineage, and substrate type are indicated for each terminal taxon. Solid

1724   black circles represent metadata from our field surveys (Supplemental Table 1; Table 1) or

1725   reference taxa (Supplemental Table 2). Solid grey circles represent metadata gathered from

1726   filtered BLASTn hits at ≥99% similarity (Supplemental Tables 6-7). Solid blue circles represent

1727   information associated with fruiting bodies, gathered by reviewing recent species monographs,

1728   species descriptions, and public databases (Supplemental Table 9). For substrate metadata, the

1729   color of outer circles indicates the condition of the host tissue at the time of collection (e.g.,

1730   asymptomatic living tissue (green), diseased living tissue (red), dead or decomposed tissue

1731   (brown), N/A or unknown (black); see substrate legend). The "Other" category for geographic

1732   location denotes isolates from Australia, New Zealand, Papua New Guinea, Africa, the Middle

1733   East, and Antarctica, which were rarely represented across the dataset.

1734

1735   **Supplemental Information**

1736

1737   **Supplemental Figure 1.** Phylogenetic relationships among 517 putative members of the

1738   Xylariomycetidae (92 endophytic and saprotrophic OTU [representing 93 total isolates] collected

1739   in our surveys and 424 previously named taxa) inferred with maximum likelihood analysis of

1740   combined ITS rDNA, LSU rDNA, *RPB2*, and β-tubulin sequences. Three species of *Ophiostoma*

1741   (Ophiostomatales, Sordariomycetes) were used to root the tree based on Huhndorf et al. (2004)

1742   and the availability of multi-locus data (Supplemental Table 2). Isolates are color-coded based on

1743   recently proposed family designations (see Senanayake et al., 2015). The main clade

1744   representing Xylariaceae plus *Graphostroma platystoma* (Graphostromataceae) and *Seynesia*

1745   *erumpens* (Cainaceae), as well as 79 endophyte and saptrotroph OTU from our surveys is

1746   collapsed for readability. See main text for discussion of *Graphostroma* and *Seynesia.* Several

1747   taxa previously classified as Xylariaceae (*Anthostomella torosa*, *Dicyma funiculosa*, and *D.*

1748   *pulvinata*) were found outside Xylariaceae. Support values are based on 1,000 maximum

1749   likelihood bootstrap replicates. Bootstrap values <50% are not shown.

1750

1751   **Supplemental Figure 2.** Probability density of phylogenetic diversity (PD; the sum of all the edge

1752   lengths in the subtree given by a subset of tips) calculated for 77 endophytic taxa and 1,000

1753   random subsets of 209 previously named Xylariaceae taxa (for a total of 286 taxa, which matches

1754   the total number of named Xylariaceae taxa used in the phylogenetic analyses; mean PD ± SD =

1755   29.5 ± 0.41). The dotted line represents the observed PD of all previously named Xylariaceae

1756   taxa without the OTU gathered in our surveys (PD = 27.91; P<0.001).

1757

1758   **Supplemental Figure 3.** Macromorphological characterization of 70 xylariaceous isolates

1759   (representing 69 OTU based on 95% ITS-partial LSU rDNA similarity) collected in our surveys

1760   from five North American sites. Cultures were grown on 2% malt extract agar (MEA) and 2%

1761   oatmeal agar (OA) under ambient light/dark condition at room temperature (ca. 21.5°C) for up to

1762   six months.

1763

1764   **Supplemental Table 1.** Geographic and host information for xylariaceous isolates collected in

1765   our surveys from five North American sites.

1766

1767   **Supplemental Table 2**. Reference taxa included in the present study.

1768

1769   **Supplemental Table 3.** Description of the single locus datasets used for analyses of the

1770   Xylariomycetidae and Xylariaceae.

1771

1772 **Supplemental Table 4**. Taxa with significant conflict (as defined in Materials and Methods)

1773 detected among single-locus phylogenies.

1774

1775 **Supplemental Table 5**. Characteristics of datasets used for the cumulative supermatrix analyses

1776 of the Xylariaceae.

1777

1778 **Supplemental Table 6**. Accession numbers for ITS rDNA sequences from 205 previously named

1779 Xylariaceae taxa not present in phylogenetic analyses due to lack of multi-locus data.

1780

1781 **Supplemental Table 7**. Metadata from ITS rDNA BLASTn hits with ≥99% identity to endophytic,

1782 endolichenic, and saprotrophic OTU collected in our surveys from five North American sites. Data

1783 reported as listed in NCBI.

1784

1785 **Supplemental Table 8**. Metadata from ITS rDNA BLASTn hits with ≥99% identity to previously

1786 named Xylariaceae taxa. Data reported as listed in NCBI.

1787

1788 **Supplemental Table 9**. Geographic, host, and substrate information for previously identified

1789 Xylariaceae taxa based on published monographs, species descriptions, or online resources.

Figure1

Figure2
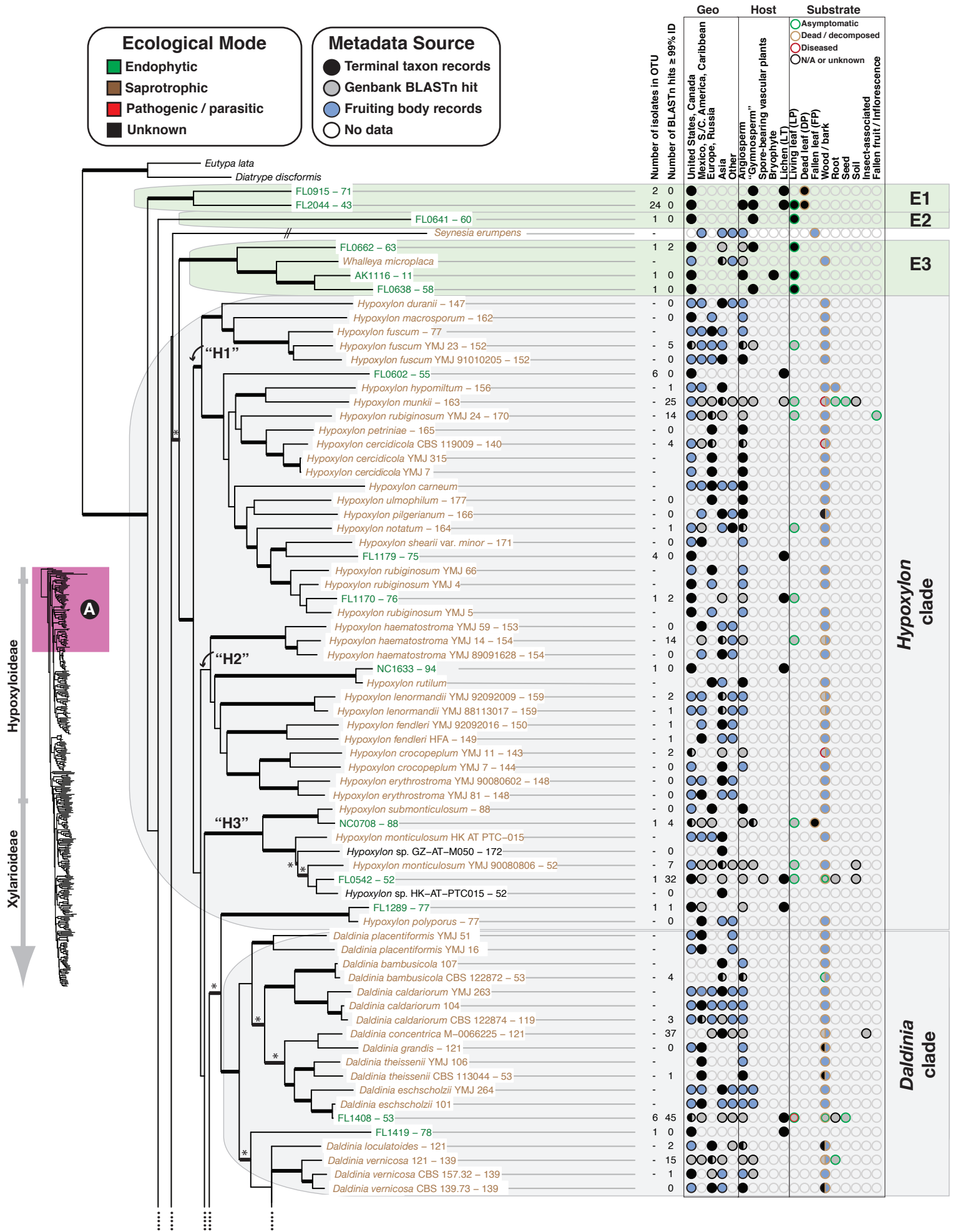
Ecological Mode
- Endophytic (green)
- Saprotrophic (brown)
- Pathogenic / parasitic (red)
- Unknown (black)

Metadata Source
- Terminal taxon records (black)
- Genbank BLASTn hit (gray)
- Fruiting body records (blue)
- No data (white)

- Asymptomatic (green outline)
- Dead / decomposed (tan outline)
- Diseased (red outline)
- N/A or unknown (black outline)

Table1

Table 1. Host species, OTU designation (95% ITS-partial LSU rDNA) and number of representatives, geographic information, and GenBank accession numbers for endophytic, endolichenic, and saprotrophic fungi collected in our surveys of five North American sites. GenBank accession numbers in bold represent new sequences generated as part of this study.

Representative Isolate	Origin*	Host species	Tissue type**	OTU (95, 100%)	Total # isolates in 95% OTU	# Isolates per tissue type (LT, LP, DP, FP)**	# Isolates per site (AZC, NCH, FLA, AK)***	Putative taxonomic classification [c]	ITS - partial LSU rDNA	RPB2	β-tubulin	α-actin
AK1199	AKN	*Equisetum arvense*	LP	10, 14	6	0, 4, 0, 2	0, 2, 0, 4	Xylariomycetidae incertae sedis	JQ759542	**submit***	**submit***	**submit***
AZ0196	AZC	*Quercus rugosa*	DP	16, 25	69	0, 14, 35, 20	42, 18, 9, 0	Xylariomycetidae incertae sedis	HM122945	**submit***	**submit***	**submit***
AZ0339	AZC	*Quercus rugosa*	FP	19, 28	13	0, 0, 1, 12	2, 7, 4, 0	Xylariomycetidae incertae sedis	HM123082	**submit***	**submit***	**submit***
FL0674	FLA	*Pinus elliottii*	LP	33, 55	6	0, 3, 2, 1	0, 0, 6, 0	Xylariomycetidae incertae sedis	JQ760367	**submit***	**submit***	**submit***
FL0456	FLA	*Cladonia didyma*	LT	46, 86	1	1, 0, 0, 0	0, 0, 1, 0	Xylariomycetidae incertae sedis	JQ760182	**submit***	**submit***	**submit***
FL0650	FLA	*Pinus elliottii*	LP	61, 248	2	0, 2, 0, 0	0, 0, 2, 0	Xylariomycetidae incertae sedis	JQ760354	**submit***	**submit***	**submit***
FL0677	FLA	*Pinus elliottii*	LP	64, 123	1	0, 1, 0, 0	0, 0, 1, 0	Xylariomycetidae incertae sedis	JQ760370	**submit***	**submit***	NA
FL0850	FLA	*Herpothallon rubrocinctum*	LT	69, 133	1	1, 0, 0, 0	0, 0, 1, 0	Xylariomycetidae incertae sedis	JQ760489	**submit***	**submit***	NA
FL1681	FLA	*Pinus clausa*	FP	80, 185	1	0, 0, 0, 1	0, 0, 1, 0	Xylariomycetidae incertae sedis	submit*	**submit***	**submit***	NA
FL1780	FLA	*Quercus inopina*	FP	82, 188	1	0, 0, 0, 1	0, 0, 1, 0	Xylariomycetidae incertae sedis	submit*	**submit***	**submit***	**submit***
FL2152	FLA	*Pinus clausa*	DP	85, 191	2	0, 0, 2, 0	0, 0, 2, 0	Xylariomycetidae incertae sedis	submit*	**submit***	**submit***	**submit***
NC0532	NCH	*Hypnum* sp.	LP	87, 205	14	3, 11, 0, 0	0, 14, 0, 0	Xylariomycetidae incertae sedis	JQ761527	**submit***	NA	**submit***
NC1491	NCH	*Pseudevernia consocians*	LT	92, 236	1	1, 0, 0, 0	0, 1, 0, 0	Xylariomycetidae incertae sedis	JQ761899	**submit***	**submit***	**submit***
FL2044	FLA	*Serenoa repens*	DP	43, 95	24	17, 1, 6, 0	0, 0, 24, 0	Xylariomycetidae incertae sedis	submit*	**submit***	**submit***	**submit***
FL0915	FLA	*Cladonia subradiata*	LT	71, 137	2	1, 0, 1, 0	0, 0, 2, 0	Xylariomycetidae sp. nov.	JQ760548	**submit***	**submit***	NA
FL0641	FLA	*Pinus elliottii*	LP	60, 120	1	0, 1, 0, 0	0, 0, 1, 0	Xylariomycetidae sp. nov.	JQ760347	**submit***	NA	**submit***
FL0662	FLA	*Pinus elliottii*	LP	63, 122	1	0, 1, 0, 0	0, 0, 1, 0	Xylariaceae sp. nov.	JQ760360	**submit***	**submit***	**submit***
FL0638	FLA	*Pinus elliottii*	LP	58, 118	1	0, 1, 0, 0	0, 0, 1, 0	Xylariaceae sp. nov.	JQ760345	**submit***	NA	**submit***
AK1116	AKN	*Cassiope tetragona*	LP	11, 15	2	0, 2, 0, 0	0, 0, 0, 2	Xylariaceae sp. nov.	JQ759464	**submit***	**submit***	NA
FL0602	FLA	*Cladonia evansii*	LT	55, 113	6	6, 0, 0, 0	0, 0, 6, 0	*Hypoxylon* sp. nov.	JQ760314	**submit***	**submit***	**submit***
FL1179	FLA	*Pyxine eschweileri*	LT	75, 161	4	4, 0, 0, 0	0, 0, 4, 0	*Hypoxylon* sp. nov.	JQ760795	**submit***	**submit***	**submit***
FL1170	FLA	*Parmotrema rampoddense*	LT	76, 159	1	1, 0, 0, 0	0, 0, 1, 0	*Hypoxylon rubiginosum*	JQ760786	**submit***	**submit***	**submit***
NC1633	NCH	*Diploschistes scruposus*	LT	94, 242	1	1, 0, 0, 0	0, 1, 0, 0	*Hypoxylon* sp. nov.	JQ761992	NA	NA	**submit***
NC0708	NCH	*Pinus strobus*	FP	88, 214	1	0, 0, 0, 1	0, 1, 0, 0	*Hypoxylon submonticulosum*	submit*	**submit***	**submit***	**submit***
FL0542	FLA	*Cladonia leporina*	LT	52, 104	1	1, 0, 0, 0	0, 0, 1, 0	*Hypoxylon monticulosum*	JQ760257	**submit***	**submit***	**submit***
FL1289	FLA	*Cladonia leporina*	LT	77, 169	1	1, 0, 0, 0	0, 0, 1, 0	*Hypoxylon polyporus*	JQ760904	**submit***	**submit***	**submit***
FL1408	FLA	*Parmotrema tinctorum*	LT	53, 111	6	6, 0, 0, 0	0, 0, 6, 0	*Daldinia eschscholzii*	JQ761025	**submit***	**submit***	NA
FL1419	FLA	*Parmotrema tinctorum*	LT	78, 179	1	1, 0, 0, 0	0, 0, 1, 0	*Daldinia* sp. nov.	JQ761035	**submit***	**submit***	**submit***
AK1016	AKE	*Arctoparmelia separata*	LT	2, 2	86	53, 25, 2, 6	0, 0, 0, 86	*Daldinia loculata*	JQ759383	**submit***	**submit***	**submit***
AZ0526	AZC	*Physcia caesia*	LT	6, 7	111	48, 38, 11, 14	1, 0, 0, 110	*Daldinia loculata*	HM123248	**submit***	**submit***	**submit***
AK0222	AKE	*Pleurozium schreberi*	LP	7, 8	29	16, 11, 1, 1	0, 0, 5, 24	*Daldinia loculata*	JQ758703	**submit***	**submit***	**submit***
AK0128	AKE	*Pleurozium schreberi*	LP	3, 3	36	17, 11, 2, 6	0, 0, 0, 36	*Daldinia loculata*	JQ758621	**submit***	**submit***	**submit***

AK0995	AKE	*Flavocetraria cucullata*	LT	4, 4	90	47, 37, 1, 5	0, 0, 0, 90	*Daldinia loculata*	JQ759362	**submit***	**submit***	**submit***
FL1857	FLA	*Pinus elliottii*	DP	83, 189	1	0, 0, 1, 0	0, 0, 1, 0	*Hypoxylon pulicicidum* (matches JX183075)	submit*	**submit***	**submit***	**submit***
NC0597	NCH	*Pseudevernia consocians*	LT	56, 114	40	33, 5, 1, 1	0, 36, 4, 0	Hypoxylon sp. nov.	JQ761586	**submit***	**submit***	**submit***
FL1377	FLA	*Parmotrema tinctorum*	LT	47, 105	26	17, 4, 2, 3	0, 19, 7, 0	Hypoxylon sp. nov.	JQ760995	**submit***	**submit***	**submit***
NC1073	NCH	*Selaginella tortipila*	LP	70, 206	9	1, 6, 2, 0	0, 8, 1, 0	Hypoxylon sp. nov.	JQ761719	**submit***	**submit***	**submit***
FL1043	FLA	*Cladonia evansii*	LT	27, 46	39	26, 10, 3, 0	0, 0, 39, 0	Hypoxylon sp. nov.	JQ760666	**submit***	**submit***	**submit***
FL0890	FLA	*Pyxine eschweileri*	LT	25, 41	98	65, 15, 7, 11	0, 0, 98, 0	Hypoxylon sp. nov.	JQ760526	**submit***	**submit***	**submit***
FL0455	FLA	*Cladonia didyma*	LT	44, 246	19	18, 0, 0, 1	0, 0, 19, 0	Annulohypoxylon sp. nov.	JQ760181	**submit***	**submit***	**submit***
FL0470	FLA	*Cladonia didyma*	LT	48, 89	4	4, 0, 0, 0	0, 0, 4, 0	*Annulohypoxylon stygium*	JQ760192	**submit***	**submit***	**submit***
FL1025	FLA	*Cladonia evansii*	LT	45, 85	19	15, 2, 1, 1	0, 7, 12, 0	*Biscogniauxia atropunctata* var. *intermedia* (matches AJ390412)	JQ760650	**submit***	**submit***	**submit***
AZ0048	AZC	*Flavoparmelia praesignis*	LT	17, 23	128	109, 10, 5, 4	89, 30, 9, 0	*Biscogniauxia mediterranea*	HM122805	NA	NA	**submit***
AZ0703	AZC	*Pseudevernia intensa*	LT	20, 31	10	8, 1, 0, 1	2, 7, 1, 0	*Biscogniauxia mediterranea*	HM123416	**submit***	**submit***	**submit***
AZ1047	AZC	*Woodsia plummerae*	LP	23, 39	2	0, 1, 0, 1	2, 0, 0, 0	*Anthostomella pinea* (matches HQ599578)	HM123694	**submit***	NA	**submit***
FL0804	FLA	*Cladonia subtenius*	LT	59, 119	5	2, 1, 0, 2	0, 0, 5, 0	Xylariaceae sp. nov.	JQ760457	**submit***	NA	**submit***
FL0016	FLA	*Aristida stricta*	LP	24, 40	1	0, 1, 0, 0	0, 0, 1, 0	Xylariaceae sp. nov.	JQ759892	**submit***	NA	**submit***
FL1105	FLA	*Cladonia subtenius*	LT	42, 74	17	13, 1, 0, 3	0, 0, 17, 0	*Muscodor vitigenus* (matches KC771503)	JQ760728	**submit***	**submit***	**submit***
FL1272	FLA	*Usnea subscabrosa*	LT	51, 125	10	6, 1, 1, 2	0, 0, 10, 0	Xylariaceae sp. nov.	JQ760887	**submit***	**submit***	**submit***
FL1255[b]	FLA	*Usnea subscabrosa*	LT	34, 83	7	7, 0, 0, 0	0, 0, 7, 0	Xylariaceae sp. nov.	JQ760870	**submit***	**submit***	**submit***
FL1019[b]	FLA	*Parmotrema perforatum*	LT	34, 66	9	6, 3, 0, 0	0, 0, 9, 0	Xylariaceae sp. nov.	JQ760644	**submit***	**submit***	**submit***
FL0660	FLA	*Pinus elliottii*	LP	62, 121	9	2, 1, 0, 6	0, 0, 9, 0	Xylariaceae sp. nov.	JQ760358	**submit***	**submit***	**submit***
FL0821	FLA	*Cladonia subtenius*	LT	67, 129	4	1, 0, 1, 2	0, 0, 4, 0	Xylariaceae sp. nov.	JQ760469	**submit***	**submit***	NA
NC1622	NCH	*Tsuga canadensis*	LP	91, 241	3	1, 2, 0, 0	0, 3, 0, 0	*Anthostomella sepelibilis* (matches AY908990)	JQ761984	**submit***	NA	**submit***
FL0255	FLA	*Pinus elliottii*	LP	26, 43	67	36, 19, 2, 10	0, 0, 67, 0	Xylariaceae sp. nov.	JQ760030	NA	**submit***	NA
FL1015	FLA	*Parmotrema perforatum*	LT	41, 81	17	16, 1, 0, 0	0, 0, 17, 0	Xylariaceae sp. nov.	JQ760640	**submit***	**submit***	**submit***
FL1651	FLA	*Quercus inopina*	FP	79, 184	1	0, 0, 0, 1	0, 0, 1, 0	*Anthostomella brabeji* (matches EU552098)[d]	submit*	**submit***	**submit***	NA
NC1498	NCH	*Xanthoparmelia conspersa*	LT	30, 237	12	7, 3, 1, 1	0, 4, 8, 0	Xylariaceae sp. nov.	JQ761906	**submit***	NA	**submit***
FL0594	FLA	*Cladonia evansii*	LT	54, 112	4	3, 1, 0, 0	0, 0, 4, 0	Xylariaceae sp. nov.	JQ760306	**submit***	**submit***	**submit***
AK1595[a]	AKE	*Salix pulchra*	DP	15, 21	1	0, 0, 1, 0	0, 0, 0, 1	Xylariaceae sp. nov.	JQ759870	**submit***	NA	NA
AK1471[a]	AKN	*Peltigera aphthosa*	LT	14, 19	7	7, 0, 0, 0	2, 1, 2, 2	*Anthostomella leucospermi* (matches EU552100)[d]	JQ759768	**submit***	**submit***	**submit***
FL0491	FLA	*Cladonia didyma*	LT	50, 94	1	1, 0, 0, 0	0, 0, 1, 0	*Xylaria* sp. nov.	JQ760210	**submit***	**submit***	**submit***
FL1030	FLA	*Cladonia evansii*	LT	66, 146	6	5, 0, 0, 1	0, 0, 6, 0	*Xylaria arbuscula*	JQ760654	**submit***	**submit***	**submit***
FL1777	FLA	*Quercus inopina*	FP	81, 187	1	0, 0, 0, 1	0, 0, 1, 0	*Xylaria* sp. nov.	submit*	**submit***	**submit***	**submit***
NC1654	NCH	*Flavoparmelia caperata*	LT	95, 244	1	1, 0, 0, 0	0, 1, 0, 0	*Kretzschmaria deusta*	JQ762008	**submit***	**submit***	**submit***
FL0490	FLA	*Cladonia didyma*	LT	49, 93	1	1, 0, 0, 0	0, 0, 1, 0	*Xylaria venustula*	JQ760209	NA	**submit***	**submit***
FL1042	FLA	*Cladonia evansii*	LT	74, 147	2	1, 0, 0, 1	0, 0, 2, 0	*Xylaria* sp. nov.	JQ760665	**submit***	**submit***	**submit***
FL0609	FLA	*Cladonia evansii*	LT	57, 116	2	2, 0, 0, 0	0, 0, 2, 0	*Xylaria* sp. nov.	JQ760320	**submit***	**submit***	**submit***

FL0359	FLA	*Pinus clausa*	LP	32, 53	38	28, 6, 2, 2	0, 9, 29, 0	*Xylaria* sp. nov.	JQ760113	**submit***	**submit***	**submit***
FL0224	FLA	*Pinus elliottii*	LP	35, 67	29	21, 7, 1, 0	0, 0, 29, 0	*Xylaria* sp. nov.	JQ760020	**submit***	**submit***	**submit***
FL0043	FLA	*Pinus elliottii*	LP	29, 50	4	3, 1, 0, 0	0, 0, 4, 0	*Xylaria* sp. nov.	JQ759911	**submit***	**submit***	**submit***
NC0985	NCH	*Flavoparmelia caperata*	LT	68, 222	31	19, 6, 4, 2	0, 15, 16, 0	*Xylaria* sp. nov.	JQ761635	**submit***	**submit***	**submit***
FL1254	FLA	*Usnea subscabrosa*	LT	37, 92	63	46, 7, 4, 6	0, 13, 50, 0	*Xylaria* sp. nov.	JQ760869	**submit***	**submit***	**submit***
FL0933	FLA	*Cladonia subradiata*	LT	40, 72	15	13, 2, 0, 0	0, 4, 11, 0	*Xylaria* sp. nov.	JQ760565	**submit***	**submit***	**submit***
FL1352	FLA	*Cladonia evansii*	LT	31, 103	71	55, 5, 6, 5	0, 13, 58, 0	*Xylaria* sp. nov.	JQ760970	**submit***	**submit***	**submit***
FL0916	FLA	*Cladonia subradiata*	LT	72, 138	1	1, 0, 0, 0	0, 0, 1, 0	*Nemania* sp. nov.	JQ760549	**submit***	**submit***	**submit***
NC1218	NCH	*Lasalia pensylvanica*	LT	90, 226	2	2, 0, 0, 0	0, 2, 0, 0	*Rosellinia subiculata* (matches AY909002)[d]	JQ761857	NA	**submit***	**submit***
NC0528	NCH	*Flavoparmelia caperata*	LT	84, 210	3	2, 0, 1, 0	0, 2, 1, 0	*Nemania* sp. nov.	JQ761524	**submit***	**submit***	**submit***
FL0031	FLA	*Pinus elliottii*	LP	28, 47	36	29, 4, 2, 1	0, 0, 36, 0	*Nemania* sp. (matches FJ175173)	JQ759904	**submit***	**submit***	**submit***
FL1152	FLA	*Parmotrema rampoddense*	LT	39, 71	15	10, 1, 2, 2	0, 0, 15, 0	*Nemania abortiva*	JQ760771	**submit***	**submit***	**submit***
NC0608	NCH	*Parmotrema reticulatum*	LT	86, 193	44	27, 6, 3, 8	0, 44, 0, 0	*Nemania diffusa*	JQ761596	**submit***	**submit***	**submit***
AK1383[a]	AKN	*Hypogymnia physodes*	LT	13, 18	1	1, 0, 0, 0	0, 0, 0, 1	*Nemania* sp. nov.	JQ759694	**submit***	**submit***	**submit***
FL0980	FLA	*Usnea mutabilis*	LT	73, 142	1	1, 0, 0, 0	0, 0, 1, 0	*Nemania beaumontii*	JQ760608	**submit***	**submit***	**submit***
FL1238	FLA	*Usnea subscabrosa*	LT	65, 127	3	2, 0, 1, 0	0, 0, 3, 0	Xylariaceae sp. nov.	JQ760853	**submit***	**submit***	**submit***
NC0429[a]	NCH	*Diploschistes scruposus*	LT	22, 199	102	67, 27, 3, 5	1, 33, 68, 0	Xylariaceae sp. nov.	JQ761458	**submit***	**submit***	**submit***
AZ0448	AZC	*Punctelia hypoleucites*	LT	21, 32	1	1, 0, 0, 0	1, 0, 0, 0	Xylariaceae sp. nov.	HM123176	**submit***	**submit***	**submit***
AZ0398[a]	AZC	*Peltigera rufescens*	LT	12, 30	74	62, 6, 2, 4	35, 32, 2, 5	*Nemania serpens*	HM123137	**submit***	**submit***	**submit***
NC0962	NCH	*Sticta beauvoisii*	LT	89, 219	1	1, 0, 0, 0	0, 1, 0, 0	*Nemania serpens* (matches AF201703)[d]	JQ761617	**submit***	**submit***	**submit***
AK0226[a]	AKE	*Pleurozium schreberi*	LP	9, 12	1	0, 1, 0, 0	0, 0, 0, 1	*Nemania serpens*	JQ758707	**submit***	**submit***	NA
NC1011	NCH	*Lecanora oreinoides*	LT	18, 27	193	154, 30, 4, 5	1, 142, 50, 0	*Xylaria cubensis*	JQ761659	**submit***	**submit***	**submit***
FL0961	FLA	*Usnea mutabilis*	LT	36, 99	61	46, 7, 3, 5	0, 23, 38, 0	*Xylaria* cf. *heliscus*	JQ760593	**submit***	**submit***	**submit***
FL0975†	FLA	*Usnea mutabilis*	LT	38, 69	36	33, 1, 0, 2	0, 5, 31, 0	*Muscodor yucatanensis* (matches FJ917287)	JQ760604	**submit***	**submit***	NA
NC1612†	NCH	*Lecanora oreinoides*	LT	93, 240	1	1, 0, 0, 0	0, 1, 0, 0	*Hypoxylon papillatum* (matches AF201710)	JQ761975	**submit***	**submit***	**submit***

* Abbreviations for sites correspond to U'Ren et al. (2012). Madrean Sky Island Archipelago of southeastern Arizona (AZC); the Appalachian Mountains of western North Carolina (NCH); subtropical scrub forest in Florida (FLA); Beringian tundra and boreal forest in the Seward Peninsula ecoregion of western Alaska (AKN); and inland, subalpine tundra in the Interior Highlands of eastern central Alaska (AKE).

** LT corresponds to lichen thallus; LP corresponds to living plant tissue; DP corresponds to dead leaves in the canopy; and FP corresponds to fallen plant leaves.

*** Isolates from AKN and AKF are pooled due to low isolation of endophytic fungi in these sites.

† Isolates were removed from the Xylariaceae mulitgene analyses due to toplogical conflict among single locus trees (Supplemental Table 4).

[a] Although not *Xylaria* spp. based on phylogenetic analyses, isolates had top BLASTn hits to *Xylaria* sp. NRRL 40192 (EF157664), which was identified based solely or

[b] Information on total isolates, isolates per tissue type, and isolates per site is based on 99% OTU.

[c] Taxonomic classification is based on sister relationship and/or shared 95% ITS rDNA OTU to reference taxa in phylogenetic tree (Fig. 2). If reference species are not present in the tree due to lack of multilocus data, identification is based on only 95% ITS rDNA OTU similarity to sequences in GenBank (denoted by accession numbers following taxon names; Supplemental Table 6).

[d] Based on our phylogeny (Fig. 2), this reference strain may be misidentified (see Supplemental Table 6).

Supplementary Tables

Supplementary Fig. 1

Click here to download Supplementary Material: SupplementalFig1_28sept2015.pdf

Supplementary Fig. 2

Click here to download Supplementary Material: SupplementalFig2_revised_10aug205.pdf

Supplementary Fig. 3