

Toward a phylogenetic classification of the Leotiomyces based on rDNA data

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Abstract: Phylogenetic relationships of one of the largest nonlichen-forming ascomycetous groups, the Leotiomyces, were inferred from genes encoding three rDNA regions (SSU+LSU+5.8S rDNA). A dataset was prepared with rDNA sequences data from 108 isolates, among which we sampled 85 taxa representing four orders and 16 families in the Leotiomyces. Equally weighted parsimony and Bayesian analyses were performed. Bootstrap proportion and Bayesian posterior probability under the GTR+ Γ +I model were estimated along the branches. Based on our results the Leotiomyces is relatively well defined as a class and it includes the Cyttariales, Erysiphales, Helotiales, Rhytismatales and two families of uncertain position, Myxotrichaceae and Pseudoeurotiaceae. The placements of the Thelebolales and Ascocorticiaceae are not examined and are accepted as tentative in the Leotiomyces. Our results agree with previous studies to remove the Geoglossaceae, including *Geoglossum*, *Trichoglossum* and *Sarcoleotia*, from the Leotiomyces. Positions of the Erysiphales and Rhytismatales in the Leotiomyces are confirmed. The Helotiales and Myxotrichaceae are paraphyletic. Close relationships are supported strongly among the Hemiphaciaceae, Rutstroemiaceae and Sclerotiniaceae, among Loramycetaceae, the northern hemisphere Vibrisseaceae, the Dark Septate Endophyte fungus *Phialocephala*

fortinii and *Mollisia cinerea*, and between species of *Bulgaria* and *Phadidiopycnis*. Sequence data of rDNA regions are not adequate to resolve the relationships among major groups of the Leotiomyces.

Key words: Ascomycetes, Geoglossaceae, Pseudoeurotiaceae

INTRODUCTION

The classification of the Ascomycota has been based traditionally on the morphology of the sporocarp (ascoma and apothecium) and ascus. The Leotiomyces includes the nonlichenized fungi producing a generally small apothecium with an exposed hymenium and an inoperculate, unitunicate ascus that has an apical perforation pore for releasing ascospores. Recent molecular studies have shown that such morphologically defined groups can be phylogenetically misleading. Several groups of fungi with simple, cleistothecial ascomata belong to the Leotiomyces, including the Erysiphales, Myxotrichaceae and Thelebolales. Conversely other groups traditionally included in the class, such as the Geoglossaceae and Orbiliaceae, have been shown to be phylogenetically distinct. Five orders, 21 families and about 510 genera (115 with an uncertain position) currently are accepted in the Leotiomyces on the basis of both traditional classification and molecular phylogenetic studies (Eriksson 2005, Kirk et al 2001).

Morphologically the Leotiomyces is a highly diverse group of the Pezizomycotina. The apothecia vary in their appearance. For example species of *Cyttaria* (Cyttariales) produce bright colored, globose fruiting bodies the size of ping-pong balls on hardwood trees, while apothecia of *Lophodermium* species (Rhytismatales) often mature as small, dark dots on conifer needles. In addition to appearance, the texture of the ascoma can range from highly gelatinous as in *Bulgaria* or hairy and fleshy to fragile as in members of the Hyaloscyphaceae. Among the Leotiomyces the fruiting bodies of the Erysiphales and Thelebolales are exceptions, with tiny, closed ascomata with one to many asci. Their position in the Leotiomyces is based mainly on molecular data. Most fungi in the Leotiomyces produce asci with eight ascospores, but more than 2000 spores can be found in a single ascus of *Thelebolus stercoreus* (Thelebolales) (de Hoog et al 2005). The ultrastructure and histochemistry of asci and ascospores has been used in some groups in the class (e.g. Baral 1987, van Brummelen 1998, Verkley 1994) but lack of

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widespread use of these characters in many groups somewhat limits the broad utility of these techniques in taxonomic or phylogenetic studies.

Characters relating to the ecology and biology of the Leotiomycetes have potential taxonomic value. Some orders, such as the Erysiphales, Cyttariales, Thelebolales and Rhytismatales, are associated with distinct ecological characters and nutritional modes in addition to morphology almost unique for each group. Members of the Helotiales, one of the largest nonlichen-forming ascomycetous groups, thrive in various ecosystems and cover a broad range of niches, and helotialean fungi have been described as plant pathogens, endophytes, nematode-trapping fungi, mycorrhizae, ectomycorrhizal parasites, fungal parasites, terrestrial saprobes, aquatic saprobes, root symbionts and wood rot fungi (Wang et al 2006). Further complicating systematics of the Helotiales are the interconnections between anamorphs and teleomorphs. Many helotialean fungi are known only from a teleomorphic stage, and their anamorphs either are undiscovered or it is assumed that they have been lost in the process of evolution. On the other hand anamorphs in various environmental samples have been suggested as members of the Helotiales without any clear teleomorph connections (Raja and Shearer <http://fm5web.life.uiuc.edu/fungi/>).

Most evolutionary studies of the Leotiomycetes are based primarily on specimens from the temperate northern hemisphere, but many members within the Leotiomycetes are patchy in their broad geographic distribution. For example there is no evidence that Sclerotiniaceae *sensu* Holst-Jensen et al (1997) occur naturally in native ecosystems of Australasia and these fungi might have evolved primarily in the northern hemisphere. However other genera such as *Cyttaria* and *Chlorovibrissa* are restricted to the southern hemisphere. Some genera such as *Chlorociboria* appear to have an Asian/Australasian center of diversity (Johnston and Park 2005), while the basal lineages of the Erysiphales seem restricted to narrow areas of South America and Asia (Takamatsu et al 2005a, b). To properly represent the genetic diversity of the Leotiomycetes requires a broad geographic focus to phylogenetic studies.

The historical classifications of the Leotiomycetes orders and families are based primarily on morphological investigations (Dennis 1968, Kimbrough 1970, Korf 1973, Nannfeldt 1932, Pfister and Kimbrough 2001). In contrast molecular phylogenetic analysis of this group of fungi is comparatively young. Several studies have revealed polyphyletic assemblages of some orders and families in the class (Gernandt et al 2001, Lutzoni et al 2004), but none of these studies have focused at the class level with

sampling from all major groups in the Leotiomycetes. The aims of this study are to (i) reconstruct the phylogeny of the Leotiomycetes based on the most inclusive rDNA dataset so far and (ii) briefly discuss phylogenetic relationships at the family level based on recognized clades in our tree and from previous studies.

MATERIAL AND METHODS

Taxon sampling and preparation of molecular data.—Sequence data of three nuclear ribosomal DNA (nuc-rDNA) regions, which were used in previous studies (Gernandt et al 2001, Goodwin 2002, Holst-Jensen et al 1997, Lumbsch et al 2005, Lutzoni et al 2004, Mori et al 2000, Rossmann et al 2004, Wang et al 2002, 2005, 2006), were downloaded from GenBank and the AFTOL databases (SUPPLEMENTARY TABLE I). Eighty-five taxa were sampled for 16 of 21 families in the Leotiomycetes accepted by Kirk et al (2001) and Eriksson (2005). A lack of adequate data excluded from our analysis the Ascocorticiaceae and Phacidiaceae (Helotiales), the Cryptomycetaceae and Ascodichaenaceae (Rhytismatales) and the Thelebolales. Species belonging to the Dothideomycetes, Lecanoromycetes, Eurotiomycetes, Orbiliomycetes, Pezizomycetes and of two budding yeasts also were sampled to address outgroup diversity. Because *Neolecta irregularis* has been suggested having a basal position in the Ascomycota (Landvik et al 2001, Liu and Hall 2004) it was used to root the tree.

Phylogenetic analyses.—A nuc-rDNA dataset of 108 taxa was prepared, which includes 1995 characters with 602 parsimony informative positions after the ambiguous or unalignable positions in the LSU-rDNA region were excluded. Sequences were aligned by eye in the data editor of PAUP* 4.0b (Swofford 1999), and the dataset was analyzed in PAUP* 4.0b and MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001) with gaps treated as missing data. Maximum parsimony analyses were based on heuristic searches of 1000 replicates of random sequence addition. MAXREES was set to auto-increase, and TBR branch swapping was employed. Bootstrap proportions (BP) were computed with a bootstrap analysis performed with 500 replicates, each with 20 random taxon addition sequences, MAXTREES was set to 1000, and TBR branch swapping was employed. Bayesian posterior probabilities were estimated with the metropolis-coupled Markov chain Monte Carlo method (MCMCMC) under the GTR+ Γ +I model in MrBayes 3.1.1 by running four chains with 2 000 000 generations. Trees were sampled every 100 generations. Likelihoods converged to a stable value after 150 000 generations, and all trees before the convergence were discarded before computing a consensus tree in PAUP*. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. Clades receiving both BP \geq 70% and PP \geq 95% were considered to be significantly supported (Lutzoni et al 2004). Alignments are available at TreeBASE (accession number sn2880).

RESULTS AND DISCUSSION

Relationships among major groups within the Leotiomycetes were investigated with three nuclear rDNA regions (LSU+SSU+5.8S) from 108 taxa. The combined genes had an aligned length of 2020 base pairs with 270 uninformative variable positions and 686 parsimony informative positions, after 25 positions were excluded from the analyses. Given the mostly unresolved or unsupported backbone the conflicts in topology between the parsimony and Bayesian analyses were interpreted as insignificant and reflected the limitations of the data. The Leotiomycetes is a large and diverse ascomycetous group, but data in GenBank remain limited and biased in terms of taxon sampling and gene sampling. We present here phylogenetic analyses based on what is thus far the most complete rDNA dataset of the Leotiomycetes.

Parsimony and Bayesian analyses.—One of the 338 equally parsimonious trees generated is shown and clades with BP \geq 50% were marked under the branches (FIG. 1). Bayesian posterior probabilities (PP) were estimated from the 50% majority rule consensus of the 18 500 trees, and clades with PP \geq 0.90 (90%) were marked above the branches (FIG. 1). The Leotiomycetes with the exclusion of the Geoglossaceae, including the Cyttariales, Erysiphales, Helotiales, Rhytismatales, Myxotrichaceae and Pseudeurotiaceae, was supported as a clade (BP = 61%, PP = 100%). The Erysiphales (BP = 63%, PP = 97%) and Rhytismatales (BP = 100%, PP = 100%) were supported as monophyletic groups. A notable exception of support (PP = 95%) of supraordinal relationships was the clade containing the *Erysiphales*, *Cyttaria darwinii* (Cyttariales) and two species of *Chlorociboria* (Helotiales). For the Erysiphales *Parauncinula septata* was supported as the most ancestral lineage with other Erysiphales as the sister group, which was composed of two clades. The one included a subclade of *Podosphaera tridactyla* and *Sawadaea polyfida* (BP = 100%, PP = 100%) with *Blumeria graminis* as the moderately supported sister group (BP = 69%, PP = 90%). The other clade (BP = 87%, PP = 100%) included two subclades that are without support. *Leveillula taurica* and *Phyllactinia moricola* were closely related (BP = 99%, PP = 100%). The Helotiales is not resolved as monophyletic, and strongly supported (BP > 70%, PP > 95%) terminal clades of two or more genera included the *Leotia*-Northern Hemisphere *Microglossum* clade (BP = 100%, PP = 100%), *Bulgaria*-Phacidiopycnis clade (BP = 98%, PP = 100%), *Ciboria*-*Sclerotinia*-*Monilinia*-*Rutstroemia*-*Sclerotium* clade (BP = 95%, PP = 100%), *Chlorenchocelia*-*Hemiphacidium*-*Heyderia*-*Fabrella*-*Rhabdocline* (teleomorph of *Meria*) clade

(BP = 97%, PP = 100%), *Dermea*-*Neofabraea* clade (BP = 84%, PP = 100%), *Cudoniella*-*Ombrophila* clade (BP = 88%, PP = 100%), *Phialocephala*-northern hemisphere *Vibrissia* clade (BP = 71%, PP = 100%), *Loramycetes*-*Mollisia* clade (BP = 99%, PP = 100%), *Chlorovibrissia*-*Vibrissia* *albofusca* clade (BP = 82%, PP = 100%) and *Ascocoryne*-*Chloroscypha* clade (BP = 95%, PP = 100%). The *Loramycetes*-*Mollisia* clade was the sister group of the *Phialocephala*-northern hemisphere *Vibrissia* clade (BP = 98%, PP = 100%), and the *Ciboria*-*Sclerotinia*-*Monilinia* clade was grouped with species of *Rutstroemia*, *Sclerotium* and *Piceomphale* (BP = 94%, PP = 100%), which was supported strongly (BP = 95%, PP = 100%) as the sister group of the *Chlorenchocelia*-*Hemiphacidium*-*Heyderia*-*Fabrella*-*Rhabdocline* clade. The Rhytismatales formed a well supported clade (BP = 100%, PP = 100%) that included the *Cudoniaceae* and members of the Rhytismataceae (*Coccomyces*, *Rhytisma* and *Lophodermium*). The Myxotrichaceae was not monophyletic in our tree and consisted of two distinct lineages. One lineage included *Byssosascus striatisporus* and *Myxotrichum deflexum* (BP = 92%, PP = 100%). The second was represented by *Pseudogymnoascus roseus*, which was part of a larger clade that included *Pseudeurotium zonatum* (Pseudeurotiaceae), *Leotia* (Leotiaceae) and *Microglossum* (Geoglossaceae).

Leotiomycetes.—The Leotiomycetes (Eriksson 2005), excluding the Geoglossaceae and including the Pseudeurotiaceae, is supported in this study as a monophyletic group. Here we accept the Leotiomycetes to include the Cyttariales, Erysiphales, Helotiales, Rhytismatales, Thelebolales, Myxotrichaceae and Pseudeurotiaceae. Two genera of the Geoglossaceae, *Geoglossum* and *Trichoglossum*, and genus *Sarcoleotia* of the Helotiaceae are removed from the class.

Cyttariales.—There is a single family, Cyttariaceae, with a single genus, *Cyttaria*, of about a dozen parasitic species on *Nothofagus* (southern beech) in the southern hemisphere. The fungus produces large and fleshy globose or pyriform stromata on galls on living branches. The stromata sometimes form spermatial pycnidia at early stages and later develop numerous separate, cup-shaped chambers containing asci and dark ascospores (Korf 1983, Pfister and Kimbrough 2001). Different authors have used morphological evidence to place the genus variously in the Pezizales and the Helotiales (see discussion in Gamundí 1991). Megoni (1986) showed that the asci have a thin cell wall and an apical amyloid ring, characters shared by some helotialean fungi. Our results placing the Cyttariales in the Leotiomycetes

agree with the Landvik and Eriksson (1994) SSU-rDNA study, and Bayesian analyses strongly support (PP = 100%) a close relationship among species of *Cyttaria* and *Chlorociboria* (Helotiales) in a clade including species of the Erysiphales. Species of *Chlorociboria* produce small disk-shaped apothecia on dead wood, and their hyphae grow inside the wood and stain the substrate blue-green. Fifteen species, including 13 new species, were reported from New Zealand based on morphological characters and ITS sequence data, and a possible Asian/Australasian center of diversity for *Chlorociboria* was suggested (Johnston and Park 2005).

Erysiphales.—The Erysiphales with one family of 15 genera (Braun et al 2002, Takamatsu et al 2005a, b) are obligate plant pathogens that cause powdery mildew diseases on about 10 000 plant species (Amano 1986). There are no direct morphological features supporting inclusion of these fungi in the Leotiomycetes. The 16 sequences from the family group into a clade with 97% of posterior probability and 63% bootstrap values. Without exception all Erysiphales have closed, nonostiolate fruit bodies (chasmothecium, Braun et al 2002) with persistent asci and basal hymenium. These characters might further support monophyly of the Erysiphales, suggesting that the morphological characters as well as the holobiotrophy were acquired in the common ancestor of the Erysiphales.

Evolutionary relationships of the Erysiphales have been based on morphological characteristics and host plants for about 100 y. They are concerned with which characters are primitive and which derived, such as (i) numbers of asci in an ascoma, (ii) number of ascospores in an ascus, (iii) morphology of appendage, (iv) conidiogenesis (i.e. conidia produced in chains [Euoidium type] or solitary [Pseudoidium type]) and (v) ecto- or endoparasitism, ectotrophic or endotrophic. Of the 15 genera of the Erysiphales, 12 are ectoparasitic and only the remaining three genera, *Leveillula*, *Phyllactinia* and *Pleochaeta*, are endoparasitic. Arnaud (1921) and Katamoto (1973) regarded the endoparasitic genus *Leveillula* as primitive and all the ectoparasitic genera derivatives from it. On the other hand Raymond (1927), Blumer (1933) and Braun (1987) argued that the ectoparasitic habit of *Leveillula* was a xerophytic adaptation and not a primitive feature. The three endoparasitic genera form a monophyletic clade in this study, suggesting that the presence of internal mycelia is a synapomorphic character for these genera, which were derived from ectoparasitic genera by a single event. Morphology of ascomatal appendages has been important to define genera of the

Erysiphales. Mori et al (2000) performed phylogenetic analyses of the Erysiphales based on the combined dataset of sequences from the same three rDNA regions as used here. They reported that the mycelioid appendage, which long has been regarded as a primitive character (e.g. Blumer 1933, Braun 1981, 1987), is a derived character of recent origin. They suggested that the development of mycelioid appendages convergently occurred many times in the respective lineages along with host expansion of the Erysiphales from trees to herbs. The basal position of *Parauncinula septata* on the present tree supports the report of Mori et al (2000) that the uncinat-circinate appendage is the most primitive in the Erysiphales.

A phylogeny covering all known tribes of the Erysiphales based on three rDNA regions by Takamatsu et al (2005a, b) suggested that *Caespitotheca forestalis* and the species of *Parauncinula septata* are basal in the order. *Caespitotheca forestalis* is restricted to South America, while *P. septata* is found only in eastern Asia.

Helotiales.—Although the backbone of our tree is not resolved, the current concept of the Helotiales (Eriksson 2005) almost certainly includes nonmonophyletic taxa. Although the Helotiales has been receiving more attention recently (e.g. Gernandt et al 2001, Lutzoni et al 2004, Wang et al 2005). Even with exclusion of groups such as the Cyttariales, Erysiphales, Myxotrichaceae, Pseudeurotiaceae, Geoglossaceae and genetically widely divergent taxa such as *Chlorociboria* and *Neobulgaria* species, the monophyly of the Helotiales is not strongly supported by rDNA data and relationships among major helotialean clades are not well resolved. No taxon represents the Ascocorticiaceae or Phacidiaceae in our tree.

The family Bulgariaceae contains only two species, *Bulgaria inquinans* and *B. nana*, which produce brown-black to black, turbinate, highly gelatinous apothecia with brown-walled ascospores on bark of living hardwoods and might be weak plant pathogens (Döring and Triebel 1998). From our results *B. inquinans* shares a clade with a weak canker-causing pathogen, *Phacidiopycnis pyri* (teleomorph: *Potebniomyces pyri*), that produces small, black, gelatinous disks from stromata beneath bark. *Potebniomyces* has been doubtfully placed in the Rhytismatales by some authors (Eriksson 2005), with DiCosmo et al (1984) suggesting a possible relationship with either Phacidiaceae, Rhytismataceae or Dermateaceae. A LSU-rDNA sequence is available for one *Phacidium lacerum* from the AFTOL database, and analyses including the sequence suggests a sister relationship between *Phacidiopycnis pyri* and *Phacidium lacerum*. This calls into doubt the morphological differences between *Potebniomyces* and

Phacidium discussed by DiCosmo et al (1984). Another black and slightly gelatinous fungus, *Holwaya mucida*, forms a sister group of the Bulgaria-Phacidiopycnis clade, although without support.

The Dermateaceae is a large, poorly studied, and heterogeneous family (Nauta and Spooner 1999, Pfister and Kimbrough 2001). Only four of the 77 genera placed in the family (Eriksson 2005) are sampled in this study. Species of *Dermea* and *Neofabraea* form a strongly supported clade, which we consider to represent the Dermateaceae *sensu stricto*. Previous studies using only the ITS region show that species of *Pezicula* and *Ocellaria* in the Dermateaceae also belong to this clade (Abeln et al 2000, de Jong et al 2001, Goodwin 2002, Verkley 1999). Species in this family produce erumpent or superficial, fleshy and small apothecia on plants, with an excipulum consisting of rounded or angular cells with dark walls. The hymenium frequently covered by a gelatinized "epithecium" and the thick walled ascospores found in several genera raise a hypothesis about their endophytic habits and the possible role of insect vectors (Wang et al unpubl). It is likely that the family will be restricted to endophytic species morphologically similar to *Dermea* and *Pezicula*. The other two genera sampled here traditionally placed in the Dermateaceae fall outside the Dermateaceae clade, *Mollisia* and *Pilidium* (teleomorph *Discohainesia*). *Mollisia* is a large, heterogeneous genus inadequately represented by the single species in our study. Crous et al (2003) discussed two phylogenetically distinct groups of *Mollisia* characterized in part by different anamorphs, while Pärtel and Raitviir (2005) described two different types of ascus ultrastructure among *Mollisia* species.

Concepts of the Geoglossaceae have changed many times recently (Eriksson 2005, Kirk et al 2001, Pfister and Kimbrough 2001, Platt 2000, Spooner 1987, Verkley 1994, Wang et al 2002, 2005). The separation of the Geoglossaceae from other helotialean fungi has been suggested, but an alternative systematic position of the family has not been proposed due to weak phylogenetic resolution (e.g. Platt 2000, Lutzoni et al 2004). Club-shaped apothecia, dark colored paraphyses and ascospores were considered as unique characters defining this group, and some authors have suggested that the hyaline-spored taxa be removed from the family (Platt 2000, Pfister and Kimbrough 2001). Species of *Geoglossum* and *Trichoglossum* form a basal branch within the inoperculate ascomycetes (Leotiomyceta) with the hyaline-spored *Sarcoleotia* species (Helotiaceae). Paraphyses (or homologous structures) cover the stipe surface in *Geoglossum* and *Trichoglossum* and obscure the boundary of the fertile hymenium (Spooner 1987).

The pileate apothecia in *Sarcoleotia globosa* have a hymenium that is continuous with the stipe at an early stage and then recedes from the stipe (Schumacher and Sivertsen 1987). The genera, such as *Cudonia*, *Spathularia*, and helotialean *Microglossum* and *Bryoglossum*, differ in that the hymenium is always clearly demarcated from the stipe, even when no pileus is formed.

The family Helotiaceae is polyphyletic in our tree, and species of the family are found in eight clades. The first of these, the Cudoniella-Hymenoscyphus clade, although not strongly supported in our tree, could form the core of a monophyletic Helotiaceae *sensu stricto*, as suggested by Pfister and Kimbrough (2001). The genus *Hymenoscyphus* might include paraphyletic lineages recognized as ericoid mycorrhizal fungi and saprobes (Monreal et al 1999, Vrålstad et al 2002, Zhang and Zhuang 2004). The second clade of the Helotiaceae, Ascocoryne-Chloroscypha, receives strong support, and species of *Neobulgaria* also might group in this clade (Wang et al unpubl). Fungi in the Ascocoryne-Chloroscypha clade are characterized by a gelatinous apothecium and endophytic habits. The third clade is unsupported statistically and includes the aero-aquatic genera *Mitrula*, *Hydrocina*, *Clathrosporium* (anamorph) and the pathogenic *Gremmeniella abietina*. An accurate phylogeny here would provide insights into the evolution of the aquatic helotialean fungi. Two helotiaceous genera, *Chlorencoelia* and *Heyderia*, are nested within the Hemiphacidiaceae. We suggest removal of these genera from the Helotiaceae to the Hemiphacidiaceae (see notes below). The other genera in our analysis that traditionally have been included in Helotiaceae are *Sarcoleotia* (included here in the Geoglossaceae), *Holwaya* (forming an unsupported clade that includes *Bulgaria*), *Chlorociboria* (see discussion under Cyttariales) and *Bisporella*.

The families Hemiphacidiaceae, Rutstroemiaceae and Sclerotiniaceae, and *Piceomphale bulgarioides*, *Heyderia abietis* and *Chlorencoelia versiformis* form a clade with high support; a new order could be an appropriate classification for this clade. The Hemiphacidiaceae traditionally contains foliar pathogens producing small, simple apothecia beneath the leaf surface, the erumpent apothecia pushing back the covering host tissue (Korf 1962). In contrast species of *Heyderia* and *Chlorencoelia* produce large apothecia with a well developed excipulum. A PCR-based study shows that *Heyderia* species colonize conifer needles as endophytes and then produce apothecia on fallen leaves (Jean Bérubé pers comm). *Chlorencoelia* and *Heyderia* are examples of significant morphological changes associated with the shift in habits from obligate pathogen to endophyte/saprobe, also illus-

SSU+LSU+5.8S nuc-rDNA,
 1995 characters with 602 parsimony-informative positions,
 Equally weighted parsimony, 1000 replicates,
 Tree 1/338, L=4285, CI=0.320, RI=0.558.

Bayesian posterior probability (>90%)
 MP bootstrap proportion(>50%)

Cytriales

- 1. Cytrariaceae

Erysiphales

- 2. Erysiphaceae

Helotiales

- 3. Bulgariaceae
- 4. Dermateaceae
- 5. Geoglossaceae (Geoglossomycetes)
- 6. Helotiaceae
- 7. Hemiphaciaceae
- 8. Hyaloscyphaceae
- 9. Leotiaceae
- 10. Loramycetaceae
- 11. Rutstroemiaceae
- 12. Sclerotiniaceae
- 13. Vibrisseaceae

Rhytismatales

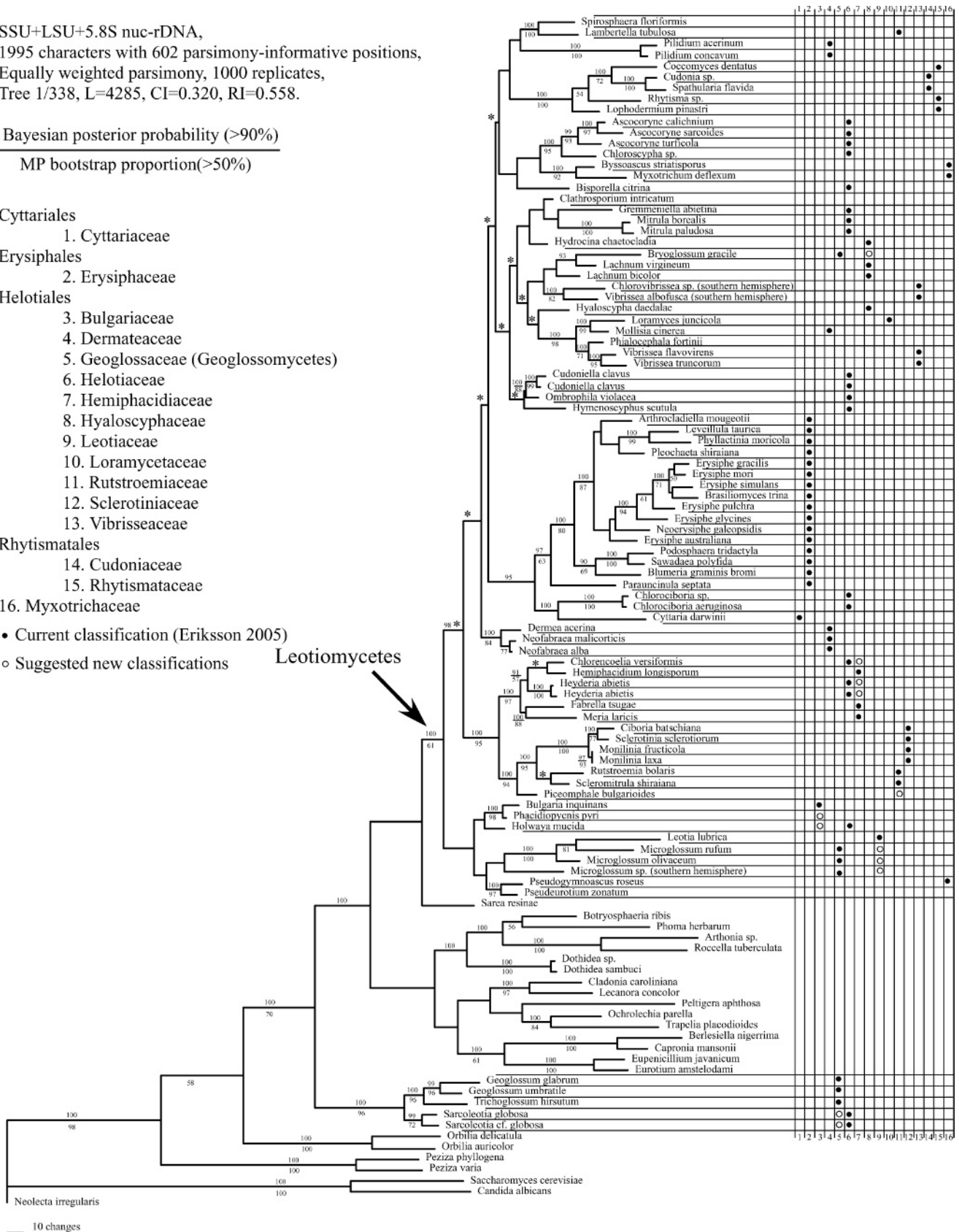
- 14. Cudoniaceae
- 15. Rhytismataceae

16. Myxotrichaceae

• Current classification (Eriksson 2005)

○ Suggested new classifications

Leotiomyces



— 10 changes

trated by the Cudoniaceae in the Rhytismatales. The Hemiphaciaceae might need to be expanded to include such genera previously placed in the Helotiaceae. Except for the angiosperm-associated *Chloroencoelia* species, fungi of the expanded Hemiphaciaceae are restricted to coniferous hosts. The Sclerotiniaceae and Rutstroemiaceae are among the best studied in the Helotiales (e.g. Dumont and Korf 1971, Holst-Jensen et al 1997, Kohn and Schumacher 1984, Novak and Kohn 1991, Schumacher and Holst-Jensen 1997, Spooner 1987, Zhuang 1998). *Piceomphale bulgarioides*, a spruce endophyte, is basal in this tree to the Sclerotiniaceae (sclerotial stromata producing) plus Rutstroemiaceae (substratal stromata producing) clade. A recent study of a chestnut pathogen *Sclerotinia pseudotuberosa* (= *Ciboria batschiana*) showed that the fungus could occur asymptotically in different tissues of the host (Vettraino et al 2005). The ancestor of this "Sclerotinales" clade was probably an endophyte living on conifers, with lineages in the Sclerotiniaceae and Rutstroemiaceae shifting from coniferous hosts to angiospermous hosts and becoming opportunistic pathogens. *Lambertella tuberosa* has been placed in the Rutstroemiaceae because of its pigmented ascospores. It has an aero-aquatic anamorph (*Helicodendron tubulosum*) and in this study it forms a clade with another aero-aquatic fungus, *Spirosphaera floriformis*, distinct from the Rutstroemiaceae. Only LSU-rDNA data were available for species of *Lambertella* and *Spirosphaera* for this study.

The Hyaloscyphaceae is poorly represented in this study, including only three of the more than 60 genera listed by Eriksson (2005). These fall in a poorly resolved part of our tree. Phylogenies from studies based on ITS sequences (Cantrell and Hanlin 1997, Abeln et al 2000, Vrålstad et al 2002) suggest the Hyaloscyphaceae subfamilies Lachnioideae and Hyaloscyphoideae are phylogenetically distinct, and Raitviir (2004) subsequently has treated these two taxa at the family level. The analysis in this paper supports removing *Bryoglossum* from the Geoglossaceae by Wang et al (2005), however the placement of *Bryoglossum* in the Hyaloscyphaceae require investigation with more inclusive taxa and gene sampling.

Habits of fungi in this family are basically unknown. Many species have been recorded as host-restricted saprobes, often an indication of an endophytic stage in their life cycle.

The Leotiaceae is represented by *Leotia lubrica* and three species of *Microglossum*, and our results support studies (e.g. Landvik 1996, Liu and Hall 2004, Platt 2000) that suggest a close relationship between *Leotia* and *Microglossum* despite morphological differences. Both *Leotia* and *Microglossum* have worldwide distribution, but the southern and northern hemisphere species of *Microglossum* are genetically distinct in our tree. The *Leotia*-*Microglossum* clade is weakly supported as forming a clade with *Pseudeurotium*, a finding however that is supported by analyses of protein coding data (Spatafora et al this volume).

The Loramyceaceae, *Mollisia cinerea*, *Phialocephala fortinii* (anamorphic dark septate endophyte), and two northern hemisphere species of the Vibrisseaceae form a strongly supported clade. Ecological linkages among these fungi support molecular evidence of evolutionary relationships between aquatic fungi and root endophytes that has been observed also within aquatic hyphomycetes (e.g. Sati and Belwal 2005). The monotypic Loramyceaceae contains only two species that are aquatic and so adapted to the aquatic environment that they morphologically are hard to classify in other families in the Helotiales. Dark cells present at the base of *Loramycetes* apothecium and the hyphal structure of the apothecia are analogous to those of the Dermateaceae (Digby and Goos 1987). Dark cells also can be found at the base of the apothecium of *Vibrissea*. Two southern hemisphere species of the Vibrisseaceae, *Chlorovibrissea* sp. and *Vibrissea albofusca*, are not included in this clade. The Vibrisseaceae is characterized by morphological features associated with an aquatic habit, and the origin of the southern hemisphere vibrisseaceous fungi could be independent from the northern hemisphere representatives. One *Mollisia* species is reported as aquatic (Fisher and Webster 1983).

Rhytismatales.—A complete rDNA dataset is available for only five of the 73 genera listed under the Rhytismatales by Eriksson (2005). Less complete datasets, or sequences from other genes, have been

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FIG. 1. Phylogenetic relationships within the Leotiomycetes inferred from three rDNA regions using parsimony analysis. One of the 338 most parsimonious trees (Length = 4285, CI = 0.320, RI = 0.558). Bootstrap values greater than 50% are indicated under nodes, branches collapsed in the strict consensus tree are marked with an asterisk. Bayesian posterior probabilities also were estimated with Bayesian approaches under the GTR+Γ+I model. Group frequencies greater than 90% are indicated above branches. New classifications suggested by molecular phylogeny are marked with open circles against solid dots as they are in current classifications.

published for several other genera either currently placed in the Rhytismatales or that have been referred to the order in the past, including *Colpoma*, *Cyclaneusma*, *Elytroderma*, *Lirula*, *Meloderma*, *Naemacyclus*, *Nematococcomyces*, *Phacidium*, *Rhabdocline*, *Rhytisma*, *Terriera*, and *Tryblidiopsis* (Gernandt et al 1997, Gernandt et al 2001, Hou et al 2004, Ortiz-Garcia et al 2003).

Many of the taxa placed in the Rhytismatales are poorly understood, with both ordinal and familial relationships of several genera remaining tentative. For most species the teleomorph comprises an apothecium with a reduced excipulum that develops within a dark stroma, the stroma typically immersed within host tissue and sometimes covered by a clypeus (Johnston 2001). The hymenium becomes exposed after the covering host and fungal tissue splits by one or more elongate slits. The asci are typically thin-walled, undifferentiated at the apex, nonamyloid, the ascospores typically with gelatinous sheaths or appendages. The anamorphs are mostly *Leptostroma*-like, the spermatial conidia developing on often percurrently proliferating conidiogenous cells. Exceptions to this typical morphology include a few genera with ascospores lacking a gelatinous sheath or appendage (e.g. *Discocainia* [Reid and Funk 1966], *Myriophacidium* [Cannon and Minter 1986], *Terriera* [Johnston 2001] and *Therrya* [Reid and Cain 1961, Yuan and Mohammed 1997] within the Rhytismataceae, *Ascodichaena* [Hawksworth and Sherwood 1982] within the Ascodichaenaceae and *Ocotomyces* [Evans and Minter 1985] incertae sedis within the Rhytismatales). The phylogenetic significance of an ascospore sheath is not yet understood, although the position of *Terriera* within the Rhytismatales is supported by ITS sequences (Ortiz-Garcia et al 2003). *Nothorhytisma* is the only genus in the order with an amyloid pore at the apex (Minter et al 1998). The presence or absence of an amyloid ascus pore has been found to be of little phylogenetic significance at higher level in discomycetes (e.g. *Rhabdocline*, see Gernandt et al 1997) although the presence of such a pore in *Phacidium* was one of the factors leading DiCosmo et al (1984) to suggest this genus was more closely related to the helotialean fungi than it was to the Rhytismatales. The exclusion of *Phacidium* from the Rhytismatales has been supported by molecular data (Gernandt et al 1997). *Cyclaneusma* and *Naemacyclus*, included in the Rhytismatales on the basis of morphology (Kirk et al 2001) and the molecular analyses of Gernandt et al (1997, 2001), showed them to be phylogenetically distinct from the Rhytismatales.

Most genera of Rhytismatales form ascomata immersed within the host tissue, but *Cudonia* and *Spathularia*, recently added to the Rhytismatales on

the basis of DNA data (Kirk et al 2001, Wang et al 2002), are different. In both genera ascomata develop on soil and form erect ascomata. The ascomata of *Cudonia* and *Spathularia* are macroscopically Helotiales-like, but careful anatomical studies have shown the hymenium covered by a stromatic layer at early stages of development (Landvik 1996, Wang et al 2002). Other Rhytismatales-like features of *Cudonia* and *Spathularia* are ascospores with gelatinous sheaths and simple asci lacking an amyloid pore (Wang et al 2002).

Available molecular data is insufficient to understand the morphological characteristics useful for defining the order Rhytismatales. Sequence data from genera such as *Lophodermium*, *Meloderma* and *Elytroderma* (Ortiz-Garcia et al 2003) suggest that generic limits within the order are artificial, but sampling intensity is too low to suggest yet what might be more appropriate generic or family limits.

Thelebolales.—A recent study of *Thelebolus* in Antarctica by de Hoog et al (2005) reveal the remarkable ecology and biology of these fungi. Species of *Thelebolus* produce small, globose to disk-shaped ascomata with sometimes polysporous asci, and the development of the ascomata varies from a cleistohymenial type to a eugymnohymenial type (van Brummelen 1967). The Thelebolaceae has been placed in the Pezizales (Eckblad 1968, Kimbrough and Korf 1967), and this placement has been studied from the apothecium macromorphology to ascus ultrastructure (e.g. Brummelen 1998). The placement of the Thelebolales in the Leotiomycetes is based on SSU-rDNA sequence data, and many of the order still have not been included in molecular phylogenetic studies (e.g. Gernandt et al 2001, Landvik et al 1998, Momol et al 1996). The order might not be monophyletic and its position in the Leotiomycetes should be regarded as a temporary treatment until sufficient proof becomes available.

Myxotrichaceae and *Pseudeurotiaceae*.—Myxotrichaceae and Pseudeurotiaceae are two families of cleistothecial ascomycetes, both of which contain genera linked to the Leotiomycetes in molecular studies (Sogonov et al 2005, Sugiyama et al 1999, Suh and Blackwell 1999). Three genera of the Myxotrichaceae are sampled in this study, and they resolve as two widely separated lineages; one includes *Byssosascus striatisporus* and *Myxotrichum deflexum* and the other includes *Pseudogymnoascus roseus*, along with *Pseudeurotium zonatum* from the Pseudeurotiaceae. The separation of *Pseudogymnoascus* from *Myxotrichum* and *Byssosascus* supports earlier studies (Sugiyama et al 1999, Sugiyama and Mikawa 2001). The link between *Pseudogymnoascus* and *Pseudeurotium* supports Sogo-

nov et al (2005). The molecular-based phylogenies are supported by electron microscope studies, with the highly reduced cleistothecial ascomata of *Myxotrichum* showing a striking similarity in morphogenesis and gross morphology to typical helotialean fungi such as *Hymenoscyphus* species (Tsuneda and Currah 2004). Sequence data of *Pseudogymnoascus* and *Pseudeurotium* are limited, and our inclusion of Pseudeurotiaceae in the Leotiomycetes is tentative. However analysis of protein coding data of *Pseudeurotium* also supported its affinity to *Leotia* (Spatafora et al this volume), although *Pseudogymnoascus* was not included in those analyses.

Conclusion.—The Leotiomycetes represents a morphologically and ecologically diverse class of Pezizomycotina whose evolutionary history is only beginning to be unraveled through phylogenetic analyses of molecular data. Significant advances have been made and include the classification of the Cudoniaceae in the Rhytismatales, the inclusion of the Erysiphales, Cyttariales and Pseudeurotiaceae in the Leotiomycetes, and the exclusion of Geoglossaceae from the Leotiomycetes. Current taxon and character sampling is insufficient to address many of the internal nodes of the class, and future phylogenetic studies must strive to significantly increase character sampling, especially that of protein-coding genes, from the diversity of species characterized as inoperculate discomycetes.

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SUPPLEMENTARY TABLE I. Species studied with information on GenBank accession numbers by DNA locus

Species	SSU-rDNA	LSU-rDNA	5.8S rDNA
<i>Arthrocladiella mougeotii</i> (Lév.) Vassilkov	AB033477	AB022379	AF073358
<i>Arthonia</i> sp.	AY571379	AY571381	AF138813
<i>Ascocoryne calichnium</i> (Tul.) Korf	AY789393	AY789394	AY789395
<i>Ascocoryne sarcoides</i> (Jacq.) J.W. Groves & D.E. Wilson	AY789387	AJ406399	AY789388
<i>Ascocoryne turficola</i> (Boud.) Korf	AY789276	AY789277	AY789278
<i>Berlesiella nigerrima</i> (R.P. Bloxam ex Curr.) Sacc.	AY541478	AY350579	AF050251
<i>Bisporella citrina</i> (Batsch.) Korf	AY789324	AY789325	AY789326
<i>Blumeria graminis</i> (DC.) Speer	AB033476	AB022362	AJ313142
<i>Botryosphaeria ribis</i> Grossenb. & Duggar	AF271129	AY004336	AF027744
<i>Brasiliomyces trina</i> (Harkn.) R. Y. Zheng	N/A	AB022350	AB022351
<i>Bryoglossum gracile</i> (P. Karst.) Redhead	AY789419	AY789420	AY789421
<i>Bulgaria inquinans</i> (Pers.) Fr.	AY789343	AY789344	AY789345
<i>Bysoascus striatisporus</i> (G.L. Barron 7 C. Booth) Arx	AJ315170	AB040688	AF062817
<i>Candida albicans</i> (C.P. Robin) Berkhout	X53497	L28817	AY672930
<i>Capronia mansonii</i> (Schol-Schwarz) E. Müll., Petrini, Fisher, Samuels & Rossman	X79318	AY004338	AF050247
<i>Chlorencoelia versiformis</i> (Pers.) Dixon	AY789350	AY789351	AY789352
<i>Chlorociboria aeruginosa</i> (Oeder) Seaver ex C.S. Ramamurthi, Korf & L.R. Batra	AY544713	AY544669	AY755360
<i>Chlorociboria</i> sp.	DQ257348	DQ257349	DQ257350
<i>Chloroscypha</i> sp.	AY544700	AY544656	U92311
<i>Chlorovibrissa</i> sp.	DQ257351	DQ257352	DQ257353
<i>Ciboria batschiana</i> (Zopf) N. F. Buchw.	DQ257354	AY789322	AY526234
<i>Cladonia caroliniana</i> (Schwein.) Tuck.	AY584664	AY584640	AF456408
<i>Clathrosporium intricatum</i> Nawawi & Kuthub.	N/A	AY616235	N/A
<i>Coccomyces dentatus</i> (J. C. Schmidt & Kunze) Sacc.	AY544701	AY544657	N/A
<i>Cudonia</i> sp.	AF107343	AF279379	AF433149
<i>Cudoniella clavus</i> (Alb. & Schwein.) Dennis	AY789340	AY789341	AY789342
<i>Cudoniella clavus</i> (Alb. & Schwein.) Dennis	AY789372	AY789373	AY789374
<i>Cyttaria darwinii</i> Berk.	U53369	UNPUBL.	UNPUBL.
<i>Dermea acerina</i> (Peck) Rehm	UNPUBL.	UNPUBL.	UNPUBL.
<i>Dibaeis baeomyces</i> (L. f.) Rambold & Hertel	AF085473	AF279385	N/A
<i>Dothidea sambuci</i> (Pers.) Fr.	AY544722	AY544681	AY883094
<i>Dothidea</i> sp.	AY016343	AY016360	AF027764
<i>Erysiphe australiana</i> (McAlpine) U. Braun & S. Takam.	N/A	AB022407	AB022408
<i>Erysiphe glycines</i> F. L. Tai	AB120748	AB022397	AB078807
<i>Erysiphe gracilis</i> R. Y. Zheng & G. Q. Chen	N/A	AB022357	AB022538
<i>Erysiphe mori</i> (I. Miyake) U. Braun & S. Takam.	AB033484	AB022418	AB000946
<i>Erysiphe pulchra</i> (Cooke & Peck) U. Braun & S. Takam.	N/A	AB022389	AB015924
<i>Erysiphe simulans</i> (E. S. Salmon) U. Braun & S. Takam.	N/A	AB022395	AB015926
<i>Eupenicillium javanicum</i> (J.F.H. Beyma) Stolk & D.B. Scott	U21298	AF263348	U18358
<i>Eurotium amstelodami</i> L. Mangin	AB002076	AY213699	AY213648
<i>Fabrella tsugae</i> (Farl.) Kirschst.	AF106015	AF356694	U92304
<i>Geoglossum glabrum</i> Pers.	AY789316	AY789317	AY789318
<i>Geoglossum umbratile</i> Sacc.	AY789302	AY789303	AY789304
<i>Gremmeniella abietina</i> (Lagerb.) M. Morelet	AF203456	UNPUBL.	U72259
<i>Hemiphacidium longisporum</i> Ziller & A. Funk	UNPUBL.	UNPUBL.	N/A
<i>Heyderia abietis</i> (Fr.) Link	AY789288	AY789289	AY789290
<i>Heyderia abietis</i>	AY789295	AY789296	AY789297
<i>Holwaya mucida</i> (Schulzer) Korf & Abawi	DQ257355	DQ257356	DQ257357
<i>Hyaloscypha daedaleae</i> Velen	AY789414	AY789415	AY789416
<i>Hydrocina chaetocladia</i> Scheuer	AY789411	AY789412	AY789413
<i>Hymenoscyphus scutula</i> (Pers.) W. Phillips	AY789430	AY789431	AY789432
<i>Lachnum bicolor</i> (Bull.) P. Karst.	AY544690	AY544674	U59005
<i>Lachnum virgineum</i> (Batsch) P. Karst.	AY544688	AY544646	U59004
<i>Lambertella tubulosa</i> Abdullah & J. Webster	N/A	AY616237	N/A

SUPPLEMENTARY TABLE I. Continued

Species	SSU-rDNA	LSU-rDNA	5.8S rDNA
<i>Lecanora concolor</i> Ramond	AY640993	AY640954	AF070037
<i>Leotia lubrica</i> (Scop.) Pers.	AY789358	AY789359	AY789360
<i>Leveillula taurica</i> (Lév.) G. Arnaud	AB033471	AB022387	AF073351
<i>Lophodermium pinastri</i> (Schrad.) Chevall	AF106014	AY004334	AF775701
<i>Loramycetes juncicola</i> W. Weston	UNPUBL.	UNPUBL.	UNPUBL.
<i>Meria laricis</i> Vuill.	AF106017	UNPUBL.	U92298
<i>Microglossum olivaceum</i> (Pers.) Gillet	AY789396	AY789397	AY789398
<i>Microglossum rufum</i> (Schwein.) Underw.	DQ257358	DQ257359	DQ257360
<i>Microglossum</i> sp.	DQ257361	DQ257362	DQ257363
<i>Mitrula brevispora</i> Zheng Wang	AY789292	AY789293	AY789294
<i>Mitrula paludosa</i> Fr.	AY789422	AY789423	AY789424
<i>Mollisia cinerea</i> (Batsch) P. Karst.	UNPUBL.	UNPUBL.	UNPUBL.
<i>Monilinia fructicola</i> (G. Winter) Honey	UNPUBL.	UNPUBL.	UNPUBL.
<i>Monilinia laxa</i> (Aderh. & Ruhland) Honey	UNPUBL.	UNPUBL.	AF150676
<i>Myxotrichum deflexum</i> Berk.	AB015777	AY541491	AF062814
<i>Neoerysiphe galeopsidis</i> (DC.) U. Braun	N/A	AB022369	AB022370
<i>Neofabraea malicorticis</i> H.S. Jacks	AY544706	AY544662	AF281386
<i>Neofabraea alba</i> (E. J. Guthrie) Velkley	N/A	AY064705	AY359236
<i>Neolecta irregularis</i> (Peck) Korf & J.K. Rogers	AY789379	AY789380	AY789381
<i>Ochrolechia parella</i> (L.) A. Massal.	AF274109	AF274097	AF329174
<i>Ombrophila violacea</i> P. Karst.	AY789364	AY789365	AY789366
<i>Orbilina auricolor</i> (A. Bloxam ex Berk.) Sacc.	AJ001986	AJ261148	U51952
<i>Orbilina delicatula</i> (P. Karst.) P. Karst.	U72603	AY261178	U72595
<i>Peltigera aphthosa</i> (L.) Willd.	AY424225	AF286759	AF158645
<i>Peziza phyllogena</i> Cooke	AY789327	AY789328	AY789329
<i>Peziza varia</i> (Hedw.) Fr.	AY789390	AY789391	AY789392
<i>Phacidiopycnis pyri</i> (Fuckel) Weindlm.	UNPUBL.	UNPUBL.	UNPUBL.
<i>Phialocephala fortinii</i> C.J.K. Wang & H.E. Wilcox	AY524846	AF269219	AY347413
<i>Phoma herbarum</i> Westend.	AY293777	AY293790	AY293802
<i>Phyllactinia moricola</i> (Henn.) Homma	AB033481	AB022401	D84385
<i>Piceomphale bulgarioides</i> (Rabenh.) Svrcek	Z81388	Z81415	Z81441
<i>Pilidium acerinum</i> (Alb. & schwein.) Kunze	AY487093	AY487092	AY487091
<i>Pilidium concavum</i> (Desm.) Höhn	AY487099	AY487098	AY487097
<i>Pleochaeta shiraiana</i> (Henn.) Kimbr. & Korf	AB120750	AB022403	D84380
<i>Podosphaera tridactyla</i> (Wallr.) de Bary	N/A	AB022393	AF154321
<i>Pseudogymnoascus roseus</i> Rallo	AB015778	AB040690	AF062819
<i>Pseudeurotium zonatum</i> J.F.H. Beyma	AF096184	AF096198	AY129286
<i>Rhytisma</i> sp.	U53370	UNPUBL.	AY465516
<i>Roccella tuberculata</i> Vain.	AF110351	AY779329	AJ634045
<i>Rutstroemia bolaris</i> (Batsch) Rehm	UNPUBL.	UNPUBL.	UNPUBL.
<i>Saccharomyces cerevisiae</i> Meyen ex E.C. Hansen	J01353	J01355	AY247400
<i>Sarcoleotia globosa</i> (Sommerf. ex Fr.) Korf	N/A	AY789409	AY789410
<i>Sarcoleotia</i> cf. <i>globosa</i>	AY789298	AY789299	AY789300
<i>Sarea resiniae</i> (Fr.) Kuntze	AY641004	AY640965	AY781237
<i>Scleromitrella shiraiana</i> (Henn.) S. Imai	AY789406	AY789407	AY789408
<i>Sclerotinia sclerotiorum</i> (Lib.) de Bary	AY789346	AY789347	AF455526
<i>Spathularia flavida</i> Pers.	AY789356	AF433142	AF433152
<i>Spirosphaera floriformis</i> Beverw.	N/A	AY616238	N/A
<i>Sawadaea polyfida</i> (C. T. Wei) R. Y. Zheng & G. Q. Chen	N/A	AB022364	AB000936
<i>Trapelia placodioides</i> Coppins & P. James	AF119500	AF274103	AF274081
<i>Trichoglossum hirsutum</i> (Pers.) Boud.	AY789312	AY789313	AY789314
<i>Uncinula septata</i> E.S. Salmon	AB183530	AB183532	AB183533
<i>Vibrissea albofusca</i> G.W. Beaton	AY789382	AY789383	AY789384
<i>Vibrissea flavovirens</i> (Pers.) Korf & J.R. Dixon	AY789425	AY789426	AY789427
<i>Vibrissea truncorum</i> (Alb. & Schwein.) Fr.	AY789401	AY789402	AY789403

Information about unpublished sequences is available from the AFTOL Web site.