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Evolution of helotialean fungi (Leotiomycetes, Pezizomycotina): A nuclear rDNA phylogeny

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Abstract

The highly divergent characters of morphology, ecology, and biology in the Helotiales make it one of the most problematic groups in traditional classification and molecular phylogeny. Sequences of three rDNA regions, SSU, LSU, and 5.8S rDNA, were generated for 50 helotialean fungi, representing 11 out of 13 families in the current classification. Data sets with different compositions were assembled, and parsimony and Bayesian analyses were performed. The phylogenetic distribution of lifestyle and ecological factors was assessed. Plant endophytism is distributed across multiple clades in the Leotiomycetes. Our results suggest that (1) the inclusion of LSU rDNA and a wider taxon sampling greatly improves resolution of the Helotiales phylogeny, however, the usefulness of rDNA in resolving the deep relationships within the Leotiomycetes is limited; (2) a new class Geoglossomycetes, including *Geoglossum*, *Trichoglossum*, and *Sarcoleotia*, is the basal lineage of the Leotiomyceta; (3) the Leotiomycetes, including the Helotiales, Erysiphales, Cyttariales, Rhytismatales, and Myxotrichaceae, is monophyletic; and (4) nine clades can be recognized within the Helotiales.

Keywords: Ascomycota; Ecology; Endophytic symbiosis; Life history; Plant pathogens

1. Introduction

The Ascomycota is the largest clade of Fungi and is characterized by the production of asci (sac-like meiosporangia producing ascospores), although asexual reproduction is common. Most species in this group are lichen-forming fungi, some are saprotrophs and parasites, and a few enter mycorrhizal associations. The classification of Ascomycota was historically based on their fruiting bodies (sporocarps or ascomata). The "discomycetes" was one of the largest and most species rich groups, but it is no longer recognized as a formal taxon (Alexopoulos et al., 1995; Kirk et al., 2001). Discomycetes develop open spore producing fruiting bodies known as apothecia, which often take on the forms of cups, saucers, cushions or clubs, and produce their asci in an exposed hymenium. Two groups of discomycetes are recognized on the basis of ascus dehiscence, those with operculate asci and those with inoperculate asci. Apothecia of inoperculate discomycetes are usually small and produce asci with an apical perforation or pore, through which the spores are discharged. Apothecia of operculate discomycetes are generally large and produce asci with a hinged cap- or lid-like structure that opens to release ascospores. Inoperculate discomycetes along with other ascomycetes producing inoperculate asci are classified in the superclass Leotiomyceta (Eriksson and Winka, 1997; Lumbsch et al., 2005), including both non-lichen- and lichen-forming fungi. These fungi colonize a large variety of habitats, and act as

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saprobes, or form parasitic associations with a wide range of other organisms. Besides parasites and saprobes, the group includes endophytes that are symbionts of a wide range of plants (Grünig and Sieber, 2005; Monreal et al., 1999; Read et al., 2000; Wilson et al., 2004).

The Helotiales in a traditional sense, which is the focus of this study, includes a polyphyletic assemblage of morphologically diverse inoperculate fungi that usually produce their ascomata not embedded in host tissue. A number of recent molecular studies have helped to improve our understanding of phylogenetic relationships in the Helotiales. For example, *Neolecta*, a former member of the Geoglossaceae that produces club-shaped sporocarps, was shown to be placed in the basal branch of Ascomycota composed of dimorphic Taphrinales parasitizing angiosperms in their mycelial stage, fission yeasts, and the mammalian pathogen *Pneumocystis carinii* (Landvik, 1996; Landvik et al., 2001; Liu and Hall, 2004). In addition, the genus *Orbilia* was shown to form a separate lineage from other inoperculate discomycetes (Baral et al. in Eriksson et al., 2003; Gernandt et al., 2001; Pfister, 1997), despite having similarly shaped fruiting bodies. Eriksson (2005) has compiled data from a wide range of recent studies, which suggest that the helotialean fungi might be closely related to several macroscopically distinct groups. He includes the Cyttariales, Erysiphales, Thelebolales, Myxotrichaceae, and Rhytismatales along with the Helotiales in the class Leotiomycetes, although these relationships remain poorly resolved (Gernandt et al., 2001; Landvik, 1996; Ogawa et al., 1997; Pfister and Kimbrough, 2001; Saenz et al., 1994).

The Helotiales includes 13 families and 395 genera, within which 92 genera are of uncertain position (Eriksson, 2005). It is the largest and the most diverse group in the Leotiomycetes, and it has already been subject to several nomenclatural reinterpretations (Carpenter, 1988; Dennis, 1968; Korf and Lizon, 2000, 2001). Most helotialean species produce small apothecia that possess relatively few characters that are diagnostic at the level of family. Morphological characters such as shape and color of the apothecia, ecological characters such as terrestrial or aquatic lifestyle, and biological characters such as parasitic or saprobic nutritional mode, have been used to define the families in the Helotiales. Species in the Helotiales, however, show extraordinary variation in these characters, and classifications based on these characters are not always consistent with cellular, ultrastructural, and molecular characters (Gernandt et al., 2001; Lutzoni et al., 2004; Verkley, 1994; Wang et al., 2005).

The morphological diversity of the Helotiales has led to the recognition of form groups, which have dominated the classification for decades (Dennis, 1968; Korf, 1973; Kirk et al., 2001). In the Helotiales, the current classification uses morphological characters such as shape and color of apothecium, hymenium, and ascospore, ontogeny of apothecia, reaction of asci to Melzer's Reagent (iodine), and ultrastructure of asci (Korf, 1973). Classifications based on apothecial morphology in this group of fungi are not always reliable, and it is likely that similar morphologies may have evolved multiple times. A good example is the Geoglossaceae, a family that includes genera with clavate or spathulate apothecia. Based on recent morphological and molecular studies, the genera of the Geoglossaceae are distributed in five different families, and the placement of the Geoglossaceae in the Helotiales has been disputed (Gernandt et al., 1997, 2001; Imai, 1941; Korf, 1973; Landvik, 1996; Lutzoni et al., 2004; Platt, 2000; Spooner, 1987; Verkley, 1994; Wang et al., 2002). Characteristic reactions of the ascus to Melzer's reagent are usually consistent within a genus but are too variable for use in higher level classification (e.g., Stone and Gernandt, 2005). The ultrastructure of asci could provide clues for inferring early relationships among ascomycetes (e.g., Baral, 1987; Verkley, 1992, 1994). However, the study of ultrastructure is technically challenging and the lack of knowledge of functions associated with observed structures limits the potential of this technique.

The systematics of the Helotiales is further hampered by a limited knowledge about interconnections between anamorphs (asexual forms) and teleomorphs (sexual forms). Many helotialean fungi are only known from a teleomorphic stage, and their anamorphs are either not yet discovered or have been lost in evolution. Anamorphs in various environmental samples including some root endophytes have been suggested to belong to the Helotiales, but without any clear teleomorph connections. In addition, there is little correlation between the classifications of helotialean teleomorphs and their anamorphs (Marvanova, 1997; Sutton and Hennebert, 1994; Raja and Shearer, http://fm5web.life.uiuc.edu/fungi/).

The overall diversity in the Helotiales makes it a focus for phylogenetic studies in the Leotiomycetes—one of the more problematic classes of Ascomycota (Lutzoni et al., 2004). Discovering more informative characters and achieving broader taxon sampling are two major challenges in phylogenetic studies of the Helotiales. Sequence data from ribosomal DNA (rDNA) have been used in phylogenetic reconstructions of major groups of ascomycetes (e.g., Berbee and Taylor, 1992; Gargas and Taylor, 1995; Eriksson and Strand, 1995; Spatafora and Blackwell, 1993) and protein-coding gene phylogenies involving helotialean fungi are slowly emerging (e.g., Landvik et al., 2001; Liu et al., 1999; Liu and Hall, 2004; Lutzoni et al., 2004). Most contemporary results suggest that the Helotiales and currently delimited families are not monophyletic, and that the highly conserved small subunit (SSU) rDNA is not informative enough to resolve these lineages with confidence (Gernandt et al., 2001). Another ribosomal locus, the internal transcribed spacers (ITS) and the 5.8S rDNA gene, has also been used to infer relationships within the Helotiales (e.g., Abeln et al., 2000; Goodwin, 2002). Closely related fungi usually form strongly supported clades in ITS phylogenies, whereas alignment difficulties make the application of ITS problematic for higher level phylogenies. For these reasons, we combined large subunit (LSU) rDNA sequences with SSU rDNA and 5.8S rDNA to estimate the phylogenetic relationships of the Helotiales.

The goals of this study were threefold: (1) to investigate the evolutionary relationships of the Cyttariales, Erysiphales, Rhytismatales, and Helotiales within the superclass Leotiomyceta using an overlapping SSU, LSU, and 5.8S rDNA data set; (2) to explore the phylogenetic structure within the Helotiales by using a diverse sample of taxa; (3) to investigate the phylogenetic distribution of morphological, biological, ecological, and biogeographic characters among the clades of the Helotiales.

2. Materials and methods

2.1. Taxon sampling

A data matrix containing 99 taxa of Pezizomycotina, 50 of them from the Helotiales, was constructed with sequences from SSU rDNA, LSU rDNA, and 5.8S rDNA genes. The data for this study were generated in laboratories at Clark University and Oregon State University, and are available from GenBank or the AFTOL database (http://ocid.nacse.org/research/aftol/data.php). Eleven of the 13 currently accepted families in the Helotiales (Eriksson, 2005) were included, excluding only the Phacidiaceae and the Ascocorticiaceae. To examine the monophyly of the Leotiomycetes and the Helotiales, species belonging to the Myxotrichaceae, Cyttariales, Rhytismatales, Erysiphales, Sordariomycetes, and Dothideomycetes were also included. *Peziza* species (Pezizomycetes), *Orbilia* species, and two budding yeasts were also sampled to address outgroup diversity. Previous studies suggested that lichen-forming inoperculate discomycetes form clades distantly related to the Leotiomycetes, thus representatives of major lichen groups were included in this study (Liu and Hall, 2004; Lumbsch et al., 2005; Lutzoni et al., 2004). *Neolecta irregularis* was suggested having a basal position in the Ascomycota (Landvik et al., 2001; Liu and Hall, 2004), and was therefore used to root the trees.

2.2. Molecular techniques

DNA was isolated from dried fruiting bodies as described in Wang et al. (2005). Crude DNA extracts were purified with GeneClean (Bio 101, La Jolla). Cleaned DNA samples were diluted with distilled water up to 500-fold for use as PCR templates. Sequence data were generated from three regions: (1) partial nuclear small subunit (SSU) rDNA bounded by primers PNS1 and NS41 (Hibbett, 1996; White et al., 1990); (2) partial nuclear large subunit (LSU) rDNA bounded by primers JS-1 and LR5 (Landvik, 1996; Vilgalys and Hester, 1990); (3) complete internal transcribed spacers 1 and 2 and the 5.8S rDNA (ITS rDNA) bounded by primers ITS-1F and ITS4 (White et al., 1990). Sequences generated in this study were submitted to GenBank, and additional sequences were downloaded from GenBank and the AFTOL database or were kindly provided by others (Table 1).

PCR mixes (Promega Corp., Madison, Wisconsin) contained 2.5 μL 10× PCR buffer, 5 μM dNTP, 12.5 pM of each PCR primer, and 5 μL DNA in 25 μL. The amplification program included 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. PCR products were purified using Pellet Paint (Novagen, Madison, Wisconsin) and sequenced using the ABI Prism BigDye-terminator cycle sequencing kit 1.1 (Applied Biosystems, Foster City, California) according to the manufacturer's protocols. Primers used for sequencing were PNS1, NS19bc, NS19b, NS41, JS-1, LR3, LR3R, LR5, ITS1F, and ITS4. Sequencing reactions were purified using Pellet Paint and were run on an Applied Biosystems 377XL automated DNA sequencer. Sequences were edited with Sequencher version 3.1 (GeneCodes Corporation, Ann Arbor, Michigan).

2.3. Phylogenetic analyses

Two data sets were prepared based on sequences of 99 taxa from three nuclear genes, SSU rDNA (950 bp), LSU rDNA (914 bp), and 5.8 S rDNA (156 bp). Four isolates of Sordariomycetes that formed a clade with very long branches in parsimony analysis (results not shown) were excluded from the final data sets. Data set one included 95 taxa and was used to resolve the phylogenetic relationships within the Helotiales and between the Helotiales and other major groups in the Leotiomycetes (wider-range analyses). Data set one contains some missing data, as follows: the SSU rDNA sequences of *Ciboria batschiana*, *Bisporella citrina*, and *Scleromitrula shiraiana* were about 360–560 base pairs (bp) shorter than sequences of the other taxa, and no SSU rDNA sequence of *Sarcoleotia globosa* was available. The LSU rDNA sequence of *Rutstroemia bolaris* was 527 bp shorter than in other taxa. No 5.8S rDNA sequences of *Hemiphacidium longisporum*, *Roccella fuciformis*, *Peltula umbilicata*, and *Dibaeis baeomyces* were available. Thirteen species were placed on conspicuously long branches and their placements were not consistent in different analyses. These problematical species include *Bisporella citrina*, *Hyaloscypha daedaleae*, *Cordierites frondosa*, *Chlorociboria* species, *Cyttaria darwinii*, three species of the Myxotrichaceae, *Byssoascus striatisporus*, *Myxotrichum deflexum*, *Pseudogymnoascus roseus*, and *Pseudeurotium zonatum* (Pseudeurotiaceae), and three species in the Erysiphales, *Arthrocladiella mougeotii*, *Blumeria graminis*, and *Uncinula septata*. Consequently, these 13 taxa and the four isolates of Sordariomycetes were excluded from data set two. Thus, data set two included 82 taxa, and was used to focus on the relationships within the Helotiales (narrower-range analyses).

Sequences were aligned with ClustalX using default setting (Thompson et al., 1997) and further adjusted by eye in the data editor of PAUP* 4.0b (Swofford, 1999). Introns were deleted and ambiguously aligned positions were excluded from the data sets before performing the analyses. All data sets were analyzed in PAUP* 4.0b

Table 1
Species studied with information on GenBank Accession numbers by DNA locus

Species	SSU-rDNA	LSU-rDNA	5.8S rDNA
Arthrocladiella mouqeotii (Lév.) Vassilkov	AB033477	AB022379	AF073358
Arthonia sp.	AY571379	AY571381	AF138813
Ascocoryne calichnium (Tul.) Korf	AY789393	AY789394	AY789395
Ascocoryne sarcoides (Jacq.) J.W. Groves and D.E. Wilson	AY789387	AJ406399	AY789388
Ascocoryne turficola (Boud.) Korf	AY789276	AY789277	AY789278
Berlesiella nigerrima (R.P. Bloxam ex Curr.) Sacc.	AY541478	AY350579	AF050251
Bisporella citrina (Batsch.) Korf	AY789324	AY789325	AY789326
Blumeria graminis (DC.) Speer	AB033476	AB022362	AJ313142
Botryosphaeria ribis Grossenb. and Duggar	AF271129	AY004336	AF027744
Bryoglossum gracile (P. Karst.) Redhead	AY789419	AY789420	AY789421
Bulgaria inquinans (Pers.) Fr.	AY789343	AY789344	AY789345
Byssoascus striatisporus (G.L. Barron 7 C. Booth) Arx	AJ315170	AB040688	AF062817
Candida albicans (C.P. Robin) Berkhout	X53497	L28817	AY672930
Capronia mansonii (Schol-Schwarz) E. Müll., Petrini, Fisher, Samuels, and Rossman	X79318	AY004338	AF050247
Chlorencoelia versiformis (Pers.) Dixon	AY789350	AY789351	AY789352
Chlorociboria aeruginosa (Oeder) Seaver ex C.S. Ramamurthi, Korf, and L.R. Batra	AY544713	AY544669	AY755360
Chlorociboria sp.	DQ257348	DQ257349	DQ257350
Chloroscypha sp.	AY544700	AY544656	U92311
Chlorovibrissea sp.	DQ257351	DQ257352	DQ257353
Ciboria batschiana (Zopf) N. F. Buchw	DQ257354	AY789322	AY526234
Cladonia caroliniana (Schwein.) Tuck	AY584664	AY584640	AF456408
Cordierites frondosa (Kobayasi) Korf	AY789353	AY789354	AY789355
Cudonia sp.	AF107343	AF279379	AF433149
Cudoniella clavus (Alb. and Schwein.) Dennis	AY789340	AY789341	AY789342
Cudoniella clavus (Alb. and Schwein.) Dennis	AY789372	AY789373	AY789374
Cyttaria darwinii Berk	U53369	UNPUBL.	UNPUBL.
Dermea acerina (Peck) Rehm	UNPUBL.	UNPUBL.	UNPUBL.
Dibaeis baeomyces (L. f.) Rambold and Hertel	AF085473	AF279385	N/A
Dothidea sambuci (Pers.) Fr.	AY544722	AY544681	AY883094
Dothidea sp.	AY016343	AY016360	AF027764
Eupenicillium javanicum (J.F.H. Beyma) Stolk and D.B. Scott	U21298	AF263348	U18358
Eurotium amstelodami L. Mangin	AB002076	AY213699	AY213648
Fabrella tsugae (Farl.) Kirschst	AF106015	AF356694	U92304
Geoglossum glabrum Pers.	AY789316	AY789317	AY789318
Geoglossum umbratile Sacc.	AY789302	AY789303	AY789304
Gremmeniella abietina (Lagerb.) M. Morelet	AF203456	UNPUBL.	U72259
Hemiphacidium longisporum Ziller and A. Funk	UNPUBL.	UNPUBL.	N/A
Heyderia abietis (Fr.) Link	AY789288	AY789289	AY789290
Heyderia abietis	AY789295	AY789296	AY789297
Holwaya mucida (Schulzer) Korf and Abawi	DQ257355	DQ257356	DQ257357
Hyaloscypha daedaleae Velen	AY789414	AY789415	AY789416
Hydrocina chaetocladia Scheuer	AY789411	AY789412	AY789413
Hymenoscyphus scutula (Pers.) W. Phillips	AY789430	AY789431	AY789432
Hypocrea lutea (Tode) Petch	AF543768	AF543791	AF359264
Lachnum bicolor (Bull.) P. Karst	AY544690	AY544674	U59005
Lachnum virgineum (Batsch) P. Karst	AY544688	AY544646	U59004
Lecanora concolor Ramond	AY640993	AY640954	AF070037
Leotia lubrica (Scop.) Pers.	AY789358	AY789359	AY789360
Lophodermium pinastri (Schrad.) Chevall	AF106014	AY004334	AF775701
Loramyces juncicola W. Weston	UNPUBL.	UNPUBL.	UNPUBL.
Meria laricis Vuill.	AF106017	UNPUBL.	U92298
Microglossum olivaceum (Pers.) Gillet	AY789396	AY789397	AY789398
Microglossum rufum (Schwein.) Underw	DQ257358	DQ257359	DQ257360
Microglossum sp.	DQ257361	DQ257362	DQ257363
Mitrula brevispora Zheng Wang	AY789292	AY789293	AY789294
Mitrula paludosa Fr.	AY789422	AY789423	AY789424
Mollisia cinerea (Batsch) P. Karst	UNPUBL.	UNPUBL.	UNPUBL.
Monilinia laxa (Aderh. and Ruhland) Honey	UNPUBL.	UNPUBL.	AF150676
Mycocalicium poplyporaeum (Nyl.) Vain	AY789361	AY789362	AY789363
Myxotrichum deflexum Berk	AB015777	AY541491	AF062814
Neobulgaria pura (Pers.) Petr	DQ257364	DQ257365	DQ257366
Neofabraea malicorticis H.S. Jacks	AY544706	AY544662	AF281386
Neofabraea alba (E. J. Guthrie) Velkley	N/A	AY064705	AY359236

Table 1 (*continued*)

Species	SSU-rDNA	LSU-rDNA	5.8S rDNA
Neolecta irregularis (Peck) Korf and J.K. Rogers	AY789379	AY789380	AY789381
Neurospora crassa Shear and B.O. Dodge	AY046271	AF286411	AF388914
Ochrolechia parella (L.) A. Massal	AF274109	AF274097	AF329174
Ombrophila violacea P. Karst	AY789364	AY789365	AY789366
Orbilia auricolor (A. Bloxam ex Berk.) Sacc.	AJ001986	AJ261148	U51952
Orbilia delicatula (P. Karst.) P. Karst	U72603	AY261178	U72595
Peltigera aphthosa (L.) Willd	AY424225	AF286759	AF158645
Peltigera degenii Gyeln.	AY584681	AF356689	AY257904
Peltula umbilicata (Vain.) Swinscow and Krog	AF356688	AF356689	N/A
Peziza phyllogena Cooke	AY789327	AY789328	AY789329
Peziza varia (Hedw.) Fr.	AY789390	AY789391	AY789392
Phialocephala fortinii C.J.K. Wang and H.E. Wilcox	AY524846	AF269219	AY347413
Phoma herbarum Westend.	AY293777	AY293790	AY293802
Piceomphale bulgarioides (Rabenh.) Svrcek	Z81388	Z81415	Z81441
Pilidium acerinum (Alb. and schwein.) Kunze	AY487093	AY487092	AY487091
Pilidium concavum (Desm.) Höhn	AY487099	AY487098	AY487097
Pseudogymnoascus roseus Raillo	AB015778	AB040690	AF062819
Pseudeurotium zonatum J.F.H. Beyma	AF096184	AF096198	AY129286
Rhytisma sp.	U53370	UNPUBL.	AY465516
Roccella fuciformis (L.) DC.	AY584678	AY584654	N/A
Roccella tuberculata Vain	AF110351	AY779329	AJ634045
Rutstroemia bolaris (Batsch) Rehm	UNPUBL.	UNPUBL.	UNPUBL.
Saccharomyces cerevisiae Meyen ex E.C. Hansen	J01353	J01355	AY247400
Sarcoleotia globosa (Sommerf. ex Fr.) Korf	N/A	AY789409	AY789410
Sarcoleotia cf. *globosa*	AY789298	AY789299	AY789300
Scleromitrula shiraiana (Henn.) S. Imai	AY789406	AY789407	AY789408
Sclerotinia sclerotiorum (Lib.) de Bary	AY789346	AY789347	AF455526
Sordaria fimicola (Roberge ex Desm.) Ces. and De Not	UNPUBL.	UNPUBL.	UNPUBL.
Spathularia flavida Pers	AY789356	AF433142	AF433152
Trapelia placodioides Coppins and P. James	AF119500	AF274103	AF274081
Trichoglossum hirsutum (Pers.) Boud	AY789312	AY789313	AY789314
Uncinula septata E.S. Salmon	AB183530	AB183532	AB183533
Vibrissea albofusca G.W. Beaton	AY789382	AY789383	AY789384
Vibrissea flavovirens (Pers.) Korf and J.R. Dixon	AY789425	AY789426	AY789427
Vibrissea truncorum (Alb. and Schwein.) Fr.	AY789401	AY789402	AY789403
Xylaria hypoxylon (L.) Grev	U20378	AF132333	AF163035

Information about unpublished sequences is available from the AFTOL website.

(Swofford, 1999) and MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001), with gaps treated as missing data.

Parsimony analyses were performed using equal weighting of characters and transformations. Heuristic searches were performed with one thousand replicate searches, each with one random taxon addition sequence, MAXTREES set to autoincrease, and TBR branch swapping. Robustness of individual branches was estimated by maximum parsimony bootstrap proportions (BP), using 500 replicate, each consisting of a single heuristic search with 50 random taxon addition sequences, MAXTREES set to autoincrease, and TBR branch swapping. Bayesian phylogenetic analyses were performed using the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC) under the GTR+Γ+I model, which was identified as the optimal model using Modeltest version 3.5 (Posada and Crandall, 1998), in MrBayes 3.1.1 by running four chains with 2,000,000 generations. Trees were sampled every 100th generation. Likelihoods converged to a stable value after ca. 500,000 generations in the wider-range analyses and after ca. 100,000 generations in the narrower-range analysis, and all trees obtained prior to 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, and clades with PP $\geqslant$ 0.95 were considered to be significantly supported.

3. Results

3.1. Phylogenetic inference from data set one (wider-range analyses)

Relationships among the Helotiales and other groups in the Leotiomycetes were investigated using three rDNA regions (LSU + SSU + 5.8S) from 95 taxa. The combined genes had an aligned length of 2020 bp (14 positions were excluded from the analyses) with 266 uninformative variable positions and 647 parsimony-informative positions.

Equally weighted parsimony analysis yielded 35 equally parsimonious trees of 4557 steps with a consistency index CI = 0.323 (Fig. 1). Although the inoperculate discomycetes were supported (BP = 70%), the backbone of the Leotiomycetes received no support. The Leotiomycetes was not

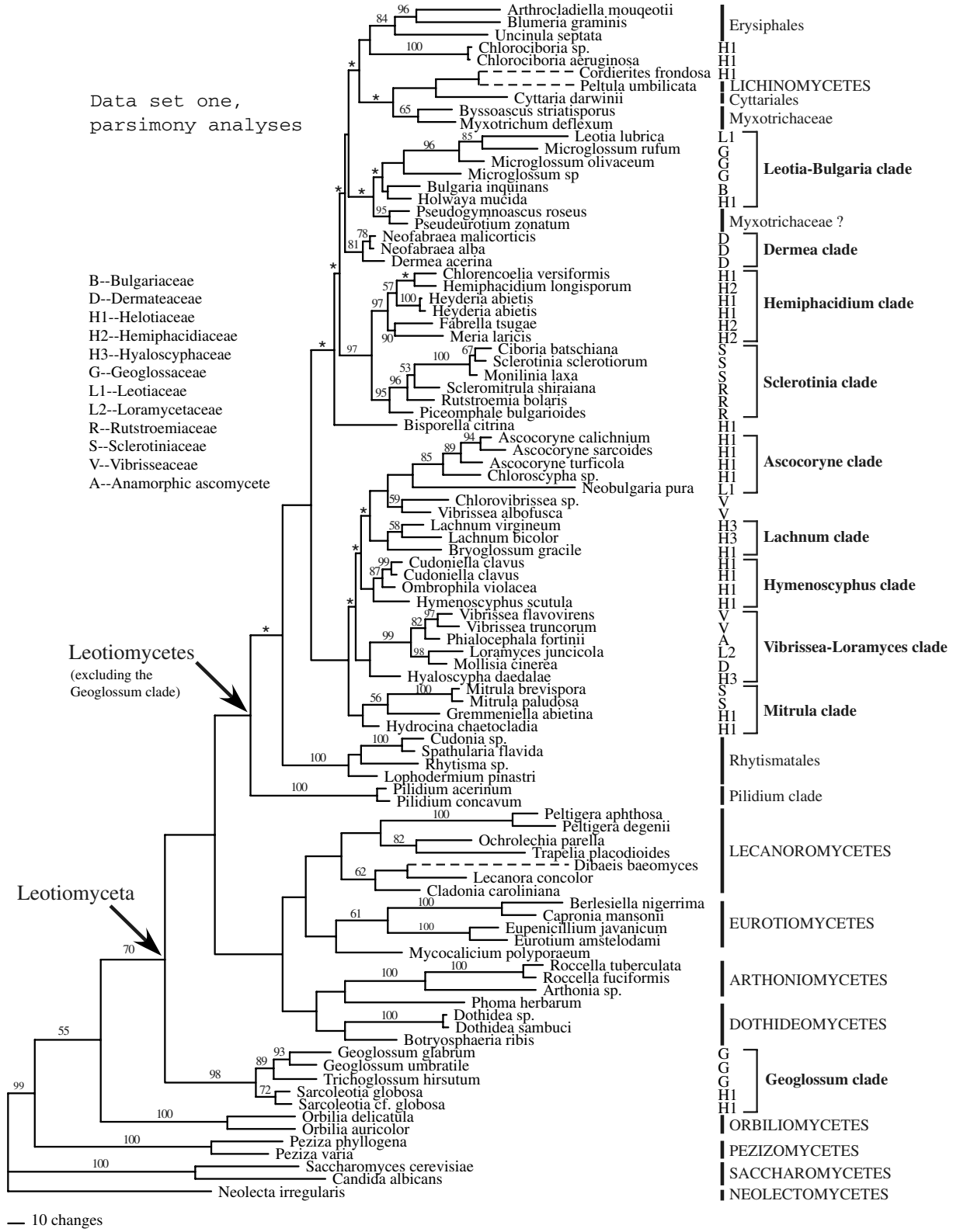

Fig. 1. Phylogenetic relationships among Helotiales and Leotiomycetes based on three rDNA regions (data set one) using parsimony analyses. Classifications follow Eriksson (2005) and family names are abbreviated and listed next to the corresponding genus. Clades discussed in this study are in boldface type. One of the 35 most parsimonious trees (Length = 4557, CI = 0.323, RI = 0.517). Bootstrap values greater than 50% are indicated along nodes, branches that collapse in the strict consensus tree are marked with asterisks. Exceedingly long branches are dashed.

monophyletic due to the placement of the Geoglossum clade (BP = 98%) including species of *Geoglossum*, *Trichoglossum*, and *Sarcoleotia*. Except for *Peltula umbilicata* (Lichinales), which apparently groups with *Corderites frondosa* possibly due to long branch attraction, members of the Dothideomycetes, Lecanoromycetes, Eurotiomycetes, and Arthoniomycetes were placed in between the Geoglossum clade and the remaining Leotiomycetes. Excluding the Geoglossum clade, the other helotialean fungi of the Cyttariales, Erysiphales, Rhytismatales, and Myxotrichaceae formed a clade with *Pilidium* (anamorph)/*Discohaninesia* (teleomorph) species as a basal branch without bootstrap support. Here, we regard this clade as the Leotiomycetes, and the Geoglossum clade was excluded from both the Helotiales and the Leotiomycetes.

The monophyly of the Helotiales was not strongly supported. Overall, the tree was not well resolved and support for the backbone of the tree was weak. Species of *Chlorociboria* (Helotiaceae) formed a clade with the Erysiphales, and *Cordierites frondosa* (Helotiaceae) formed a clade with *Peltula umbilicata* (Lichinales) and *Cyttaria darwinii* (Cyttariales), however, these relationships were not supported by bootstrap values. Relationships among the Helotiales, Erysiphales, Cyttariales, and Myxotrichaceae were not resolved. Although most families in the Helotiales were not monophyletic, some clades can be recognized with substantial support within the Helotiales: (1) the Dermea clade, including three species in the Dermateaceae, *Neofabraea malicorticis*, *N. alba*, and *Dermea acerina*, formed a lineage (BP = 81%) with an unresolved position in the strict consensus tree. (2) The Hemiphacidium clade, including three species of the Hemiphacidiaceae, *Hemiphacidium longisporum*, *Fabrella tsugae*, and *Meria laricis*, and two species of the Helotiaceae, *Chlorencoelia versiformis* and *Heyderia abietis*, was strongly supported (BP = 97%). (3) The Sclerotinia clade, including a subclade (BP = 100%) of three species of the Sclerotiniaceae, *Ciboria batschiana*, *Sclerotinia sclerotiorum*, and *Monilinia laxa*, and two species in the Rutstroemiaceae, *Scleromitrula shiraiana* and *Rutstroemia bolaris*, and *Piceomphale bulgarioides*, received strong support (BP = 95%). (4) The Ascocoryne clade included species of *Ascocoryne*, *Chloroscypha*, and *Neobulgaria pura* on a long branch, but *Ascocoryne* and *Chloroscypha* species were closely related (BP = 85%). (5) The Lachnum clade composed of two *Lachnum* species (BP = 58%) and *Bryoglossum gracile* was not supported. (6) The Hymenoscyphus clade including *Cudoniella clavus* and *Ombrophila violacea*, was supported (BP = 87%), with *Hymenoscyphus scutula* as the sister group. (7) The Vibrissea-Loramyces clade was strongly supported (BP = 99%), and within the clade, close relationships between *Vibrissea* and *Phialocephala* (BP = 82%), and between *Loramyces* and *Mollisia* (BP = 98%) received support. (8) The Mitrula clade included a weakly supported group (BP = 56%) of *Mitrula* species and *Gremmeniella abietina*, and *Hydrocina chaetocladia*. (9) The Leotia-Bulgaria clade, including species of *Leotia*, *Microglossum*, *Bulgaria*, and *Holwaya*, collapsed in the strict consensus tree, however, these four genera and two species of the Myxotrichaceae were grouped together by all analyses. A clade including *Leotia lubrica*, *Microglossum rufum*, and *M. olivaceum* collected from the Northern Hemisphere was supported (BP = 96%), with a *Microglossum* species from New Zealand as the sister group (BP < 50%). Relationships among those nine clades were not resolved, except for a sister relationship between the Hemiphacidium clade and the Sclerotinia clade (BP = 97%).

There was no significant conflict between the results of the Bayesian analysis of data set one (Fig. 2) and the results from parsimony analyses, however, support for the clades and deeper nodes of the tree from Bayesian analyses were generally higher. The Geoglossum clade received strong support (PP = 1.0), and its basal position within the superclass Leotiomyceta was upheld (PP = 1.0). The Lecanoromycetes, Eurotiomycetes, Arthoniomycetes, and Dothideomycetes were all supported as monophyletic groups (PP = 1.0), but the relationships among those groups received no support (PP = 0.53–0.77).

The Leotiomycetes were supported as monophyletic with PP = 1.0. The Helotiales was not resolved as monophyletic. *Chlorociboria* species shared a clade with the Cyttariales and the Erysiphales (PP = 0.98), and *Cordierites frondosa* shared a clade with the Myxotrichaceae (PP = 1.0). Within the Helotiales, clades recognized in the parsimony analysis were recovered in the Bayesian analysis, even though support for the backbone of this part of the tree was weak (PP = 0.53–0.88). Contents of the Dermea clade (PP = 1.0), Hemiphacidium clade (PP = 1.0), Lachnum clade (PP = 0.95), Ascocoryne clade (PP = 1.0), Sclerotinia clade (PP = 1.0), and Mitrula clade (PP = 0.98), were the same as in the parsimony analysis, but received much stronger support. The Vibrissea-Loramyces clade was strongly supported (PP = 1.0), and within the clade, close relationships between *Vibrissea* and *Phialocephala*, and between *Loramyces* and *Mollisia* were confirmed with PP = 1.0. Two New Zealand isolates, *Chlorovibrissea* sp. and *Vibrissea albofusca*, formed a lineage sister to the Vibrissea-Loramyces clade without strong support (PP = 0.86). The Leotia-Bulgaria clade was not resolved in the Bayesian analysis, and there was no support for a clade including *Leotia lubrica* and all *Microglossum* species. Relationships among the helotialean clades were not resolved with statistic support, except for the sister relationship between the Hemiphacidium clade and the Sclerotinia clade (PP = 1.0).

3.2. Phylogenetic inference from data set two (narrower-range analyses)

Relationships within the Helotiales were examined using three rDNA regions (LSU + SSU + 5.8S) from 82 taxa, with an aligned length of 2020 bp (14 were excluded from the analyses) including 242 uninformative variable positions and 628 parsimony-informative positions.

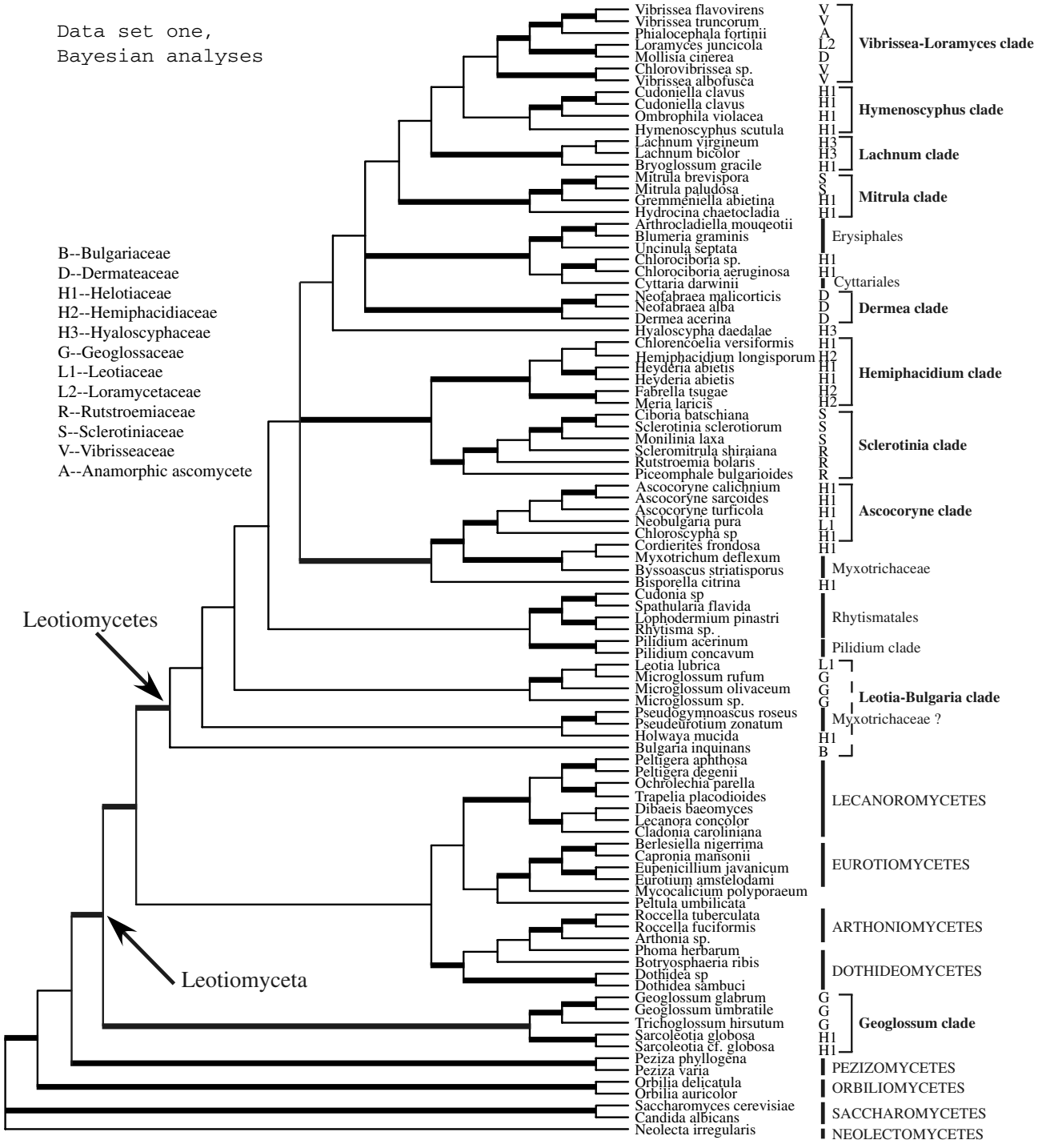

Fig. 2. Phylogenetic relationships among the Helotiales and the Leotiomycetes inferred from three rDNA regions (data set one) using Bayesian approaches under the GTR+Γ+I model. Classifications follow Eriksson (2005), and family names are abbreviated and listed next to the corresponding genus. Majority-rule consensus tree of 19,000 MCMCMC-sampled trees. Group frequencies greater than 0.95 are indicated as bold branches.

Equally weighted parsimony analysis yielded 69 equally parsimonious trees of 3964 steps and consistency index CI = 0.349 (Fig. 3). The strict consensus tree based on the 69 trees was much better resolved than the one based on the 35 trees in the wider-range analyses. The Geoglossum clade (BP = 99%) formed the basal branch within the Leotiomyceta (BP = 67%). The Helotiales was monophyletic (BP < 50%). The Hemiphacidium clade (BP = 97%) was composed of a subclade (BP = 87%) of *Fabrella tsugae* and *Meria laricis*, a subclade (BP = 53%) of *Chlorencoelia versiformis* and *Heyderia abietis*, and *Hemiphacidium longisporum*. The Sclerotinia clade (BP = 93%) included species of *Scleromitrula*, *Rutstroemia*, *Piceomphale*, and a subclade of *Ciboria batschiana*, *Sclerotinia sclerotiorum*, and *Monilinia*

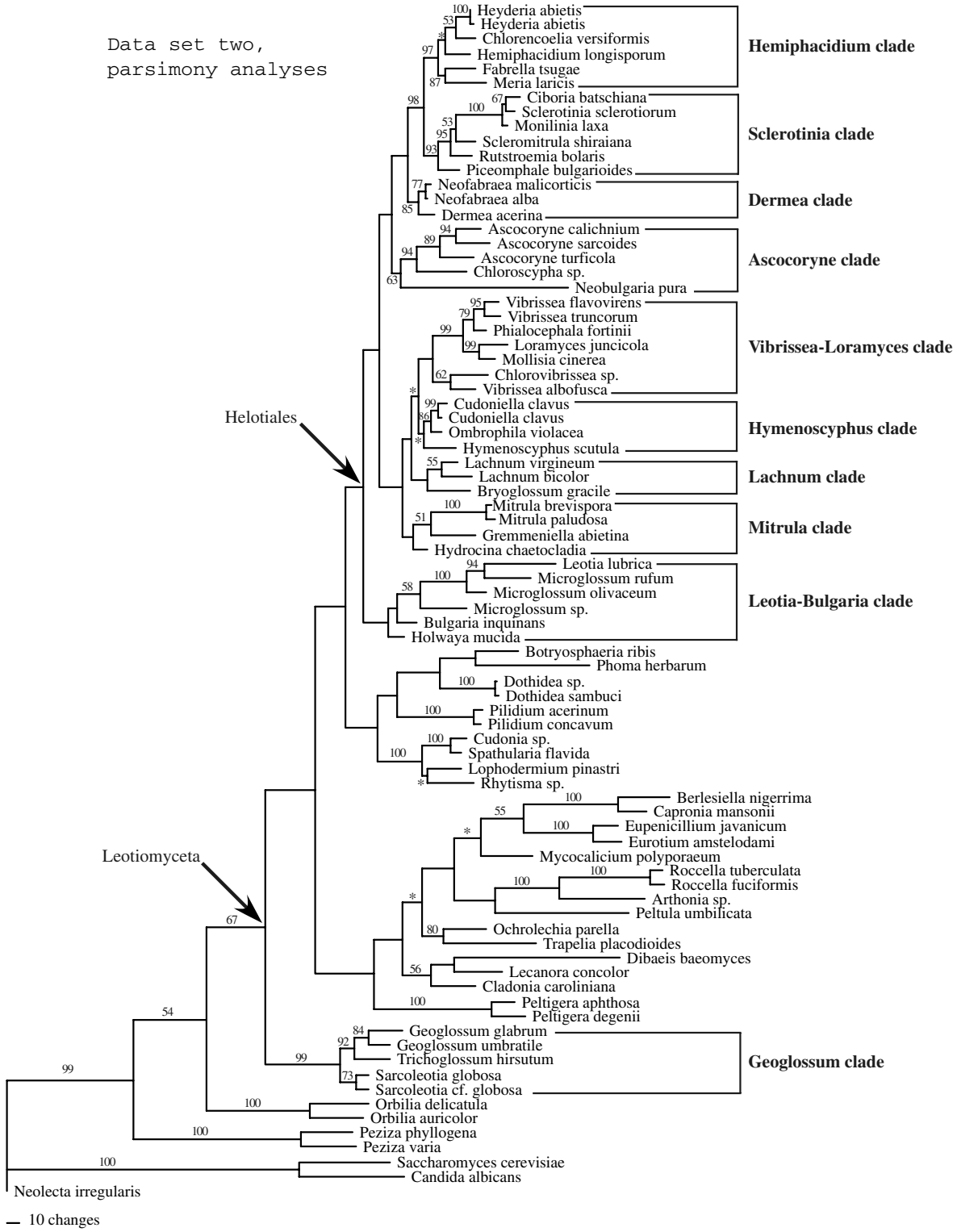

Fig. 3. Phylogenetic relationships within the Helotiales inferred from three rDNA regions (data set two) using parsimony analysis. One of the 69 most parsimonious trees (Length = 3964, CI = 0.349, RI = 0.539). Bootstrap values greater than 50% are indicated along nodes, branches that collapse in the strict consensus tree are marked with asterisks.

laxa (BP = 100%). The Dermea clade (85%) included a subclade of *Neofabraea* species (77%) and *Dermea acerina*. The Ascocoryne clade (BP = 63%) was weakly supported with *Neobulgaria pura* as the sister lineage to the core clade including species of *Ascocoryne* and *Chloroscypha* (BP = 94%). The Vibrissea-Loramyces clade (BP < 50%) was composed of a southern lineage (99%) of *Chlorovibrissea* sp. and *Vibrissea albofusca*, and a northern lineage (99%) including two clades: one clade of *Vibrissea* species and *Phialocephala fortinii* (BP = 79%), and another of

Loramyces juncicola and *Mollisia cinerea* (99%). The Hymenoscyphus clade collapsed in the strict consensus tree, but the close relationship between *Cudoniella* and *Ombrophila* species was supported (BP = 86%). The Lachnum clade was not supported (BP < 50%) and *Lachnum* species received weak support (BP = 55%). The Mitrula clade, including species of *Mitrula*, *Gremmeniella*, and *Hydrocina chaetocladia*, received no support (BP < 50%). The Leotia-Bulgaria clade was resolved in the strict consensus tree, and the New Zealand *Microglossum* species clade was weakly supported (BP = 51%) as the sister group to a subclade including the northern collections of *Leotia* and *Microglossum* (BP = 100%). The sister group relationship between the Hemiphacidium clade and the Sclerotinia clade was highly supported (98%). The Leotia-Bulgaria clade was positioned as the basal branch in the Helotiales in this analysis without support. The Vibrissea-Loramyces clade, Hymenoscyphus clade, Lachnum clade and the Mitrula clade formed a monophyletic group without support (BP < 50%).

4. Discussion

4.1. Limits and relationships of the Helotiales in the Leotiomycetes

Both the wider-range and narrower-range analyses suggest that *Geoglossum* species and related fungi form a basal lineage in the Leotiomyceta, and that the relationship between this lineage and other members of the Leotiomycetes is distant. This result agrees with previous studies in separating the Geoglossum clade from other Leotiomycetes (e.g., Lutzoni et al., 2004; Reeb et al., 2004). However, conflicts in the systematic position of the Geoglossum clade remain.

The remainder of the Leotiomycetes, which includes the Cyttariales, Helotiales, Erysiphales, Rhytismatales, and the Myxotrichaceae, was supported as a monophyletic group in both wider- and narrower-range analyses. Although the majority of relationships within the Leotiomycetes were not resolved with strong statistical support, the Erysiphales and the Rhytismatales were strongly supported as monophyletic. Studies based on ascocarp development and rDNA phylogenies suggested a placement of the Myxotrichaceae in the inoperculate ascomycetes (Sugiyama and Mikawa, 2001; Tsuneda and Currah, 2004), and our results support including this family in the Leotiomycetes. However, monophyly of the Myxotrichaceae is not supported in this study, and more data are needed to examine the relationships between the Myxotrichaceae, Pseudeurotiaceae, and saprotrophic helotialean fungi.

The most surprising relationship within the Leotiomycetes is a clade including the Erysiphales, Cyttariales, and *Chlorociboria* species (Figs. 1 and 2). Given the striking difference in macromorphology between these fungi, this relationship could be an artifact of insufficient informative characters, and/or unbalanced taxon sampling. Nevertheless, some significant aspects of these fungi are worth mentioning here. The Erysiphales is one of the most intensively studied groups of the Leotiomycetes since they are obligate plant pathogens, causing powdery mildew diseases on plant species (Matsuda and Takamatsu, 2003). Species of the Erysiphales reproduce sexually by means of ascospores within asci in completely closed, minute ascocarps on leaves, and there are no morphological features supporting the molecular data linking these fungi to the Leotiomycetes (Gargas and Taylor, 1995). Some lineages of the Erysiphales apparently have a geographic origin in the Southern Hemisphere, with subsequent dispersal throughout the Northern Hemisphere (Bremer, 1994; Takamatsu and Matsuda, 2004). The Cyttariales, containing a single genus, *Cyttaria*, is composed of about a dozen species. *Cyttaria* species are parasites on the Southern Hemisphere beech, *Nothofagus*, in southern South America, Australia, and New Zealand (Gamundí, 1991). The systematic position of the Cyttariales remains unclear, with inconsistent results from morphological studies and molecular phylogenies (Carpenter, 1976; Korf, 1983; Landvik, 1996). *Chlorociboria* species generally produce a blue-green staining on fallen wood. 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 the *Chlorociboria* was suggested (Johnston and Park, 2005).

With the Erysiphales, Cyttariales, Myxotrichaceae, and species of *Chlorociboria* and *Cordierites frondosa* excluded, results from the narrower-range analyses supported the Helotiales as a monophyletic group with the Rhytismatales and *Discohaninesia*/*Pilidium* (traditionally placed in the Helotiales family Dermateaceae) as the sister group (Figs. 3 and 4).

4.2. Phylogenetic and ecological diversity of the Helotiales

The limited sampling and the poorly resolved phylogenetic relationships in this study make it premature to present a revised taxonomy of the Helotiales. Classification is an important prerequisite for the ecological and biological study of organisms, and the major purpose of this study is to provide a framework for future phylogenetic classifications. With a few exceptions, our results are more or less congruent with the current classification of the Helotiales at a higher level (Eriksson, 2005). Some clades are not strongly supported by molecular characters, and in these cases, characters of morphology, ecology, and biology are used to define the clade.

4.2.1. Phylogenetic distribution of ecological and biological characters

Biological relationships of helotialean fungi in ecosystems are diverse, and members of the Helotiales have been described as plant pathogens, endophytes, nematode-trapping fungi, mycorrhiza-forming (including ectomycorrhizae and ericoid mycorrhizae), ectomycorrhizal parasites, fungal parasites, terrestrial saprobes, aquatic saprobes, root

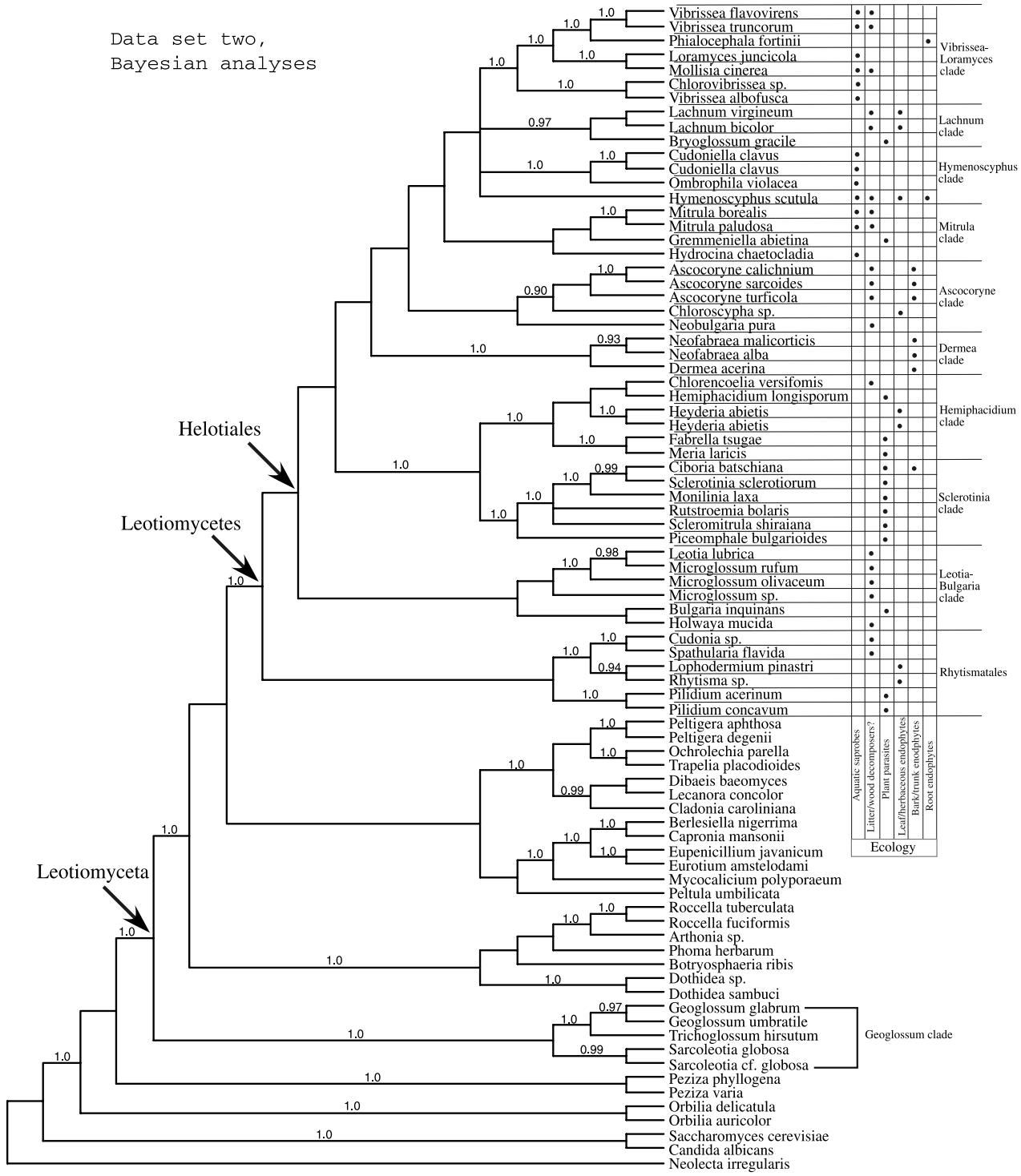

Fig. 4. Phylogenetic relationships within the Helotiales based on three rDNA regions (data set two) using Bayesian approaches under the GTR+Γ+I model. The majority-rule consensus of 19,000 MCMCMC-sampled trees. The resulting posterior probabilities (PP) greater than 0.90 are shown above branches.

symbionts, and wood rot fungi (Boddy, 2001; Grünig et al., 2002; Grünig and Sieber, 2005; Hosoya and Otani, 1995; Johnston and Park, 2005; Monreal et al., 1999; Platt, 2000; Pöder and Scheuer, 1994; Shoemaker et al., 2002).

Endophytes represent putative symbiotic interactions between fungi and plants and live within plant tissues without producing noticeable symptoms. Endophytic fungi have been found in various vegetative organs and from a broad range of plant hosts, and they can influence the distribution, ecology, and biology of plants (Arnold et al., 2003; Carroll, 1988; Sridhar and Raviraja, 1995). Fungi termed endophytes have a wide range of lifestyles

(Stone et al., 2004) and amongst the Leotiomycetes include the Sclerotiniaceae, Rutstroemiaceae, Hemiphacidiaceae, Phacidiaceae, the Hyaloscyphaceae, Dermateaceae, Bulgariaceae, and Helotiaceae in the Helotiales, as well as the Rhytismatales, Erysiphales, and probably the Cyttariales (Fig. 4) (Egger and Sigler, 1993; Johnston, 1989; Platt, 2000; Rossman et al., 2004; Vrålstad et al., 2002a,b; Wilson et al., 2004). Such a broad distribution of the endophytic lifestyle suggests it could be plesiomorphic in the Helotiales, but inadequate information of biology and poorly resolved phylogeny within the Leotiomycetes make it premature to reconstruct the ancestral lifestyle in the Helotiales.

Fungal endophytes are mainly ascomycetes, and the endophytic lifestyle may play an important role in the evolution of the higher ascomycetes. Endophytes are able to colonize host tissue early and occupying the habitat puts them in a good position to make a shift to parasitism (when hosts are under stress) or to saprophytism (after hosts die). Many helotialean fungi are collected from fallen leaves, dead ferns, and herbaceous debris, and were recorded as saprobes, whereas current studies using molecular probes suggest that at least some of them have endophytic stages or are closely related to endophytes (Abeln et al., 2000; Cabral, 1985; Johnston, 1998; Monreal et al., 1999).

4.2.2. Clades

The clades discussed below are named after the representative genera as well as important morphological, ecological, and/or biological characters (in parentheses). Biological relationships among helotialean clades are discussed on the basis of the rDNA phylogeny.

4.2.2.1. Geoglossum clade (black terrestrial saprobe clade)—Geoglossomycetes. Species of the genera *Geoglossum*, *Trichoglossum*, and *Sarcoleotia* are included in this clade. The concept of the Geoglossaceae has been changed and modified recently (Eriksson, 2005; Pfister and Kimbrough, 2001; Platt, 2000; Spooner, 1987; Wang et al., 2002, 2005). The separation of the Geoglossaceae from other helotialean fungi has been suggested in previous studies, and paraphyses with dark pigments and dark ascospores with multiple septa were considered as unique characters defining this group (Platt, 2000; Lutzoni et al., 2004). Our results suggest a clade including species of *Geoglossum*, *Trichoglossum*, and *Sarcoleotia* are holding the basal position in the superclass Leotiomyceta with strong support, and a new class, the Geoglossomycetes, is proposed for this clade.

Color and the number of septa in the ascospores of *Geoglossum* and *Trichoglossum* are variable among species and with ascus age (Zhuang, 1998), and thus should not be considered as a consistent morphological character for this clade. *Sarcoleotia globosa* produces pileate, black apothecia, and hyaline ascospores with 0–5 septa, and has been included in the Helotiaceae (Schumacher and Sivertsen, 1987). Paraphyses (or homologous structures) cover the stipe surface in *Geoglossum* and *Trichoglossum*, and obscure the boundary of the fertile hymenium (Spooner, 1987), a phenomenon not known from other inoperculate ascomycetes. Similar to *Geoglossum*, the pileate apothecia in *S. globosa* have a hymenium that is continuous with the stipe at an early stage, and then recedes from the stipe to form a pileate like fruit body (Schumacher and Sivertsen, 1987). This differs from other helotialean fungi with pileate apothecia such as species of *Cudoniella* and *Leotia*, which have a hymenium that is bounded by the edge of the excipulum. Species of *Microglossum*, *Thuemenidium*, and *Bryoglossum* also have a distinct hymenium boundary, and this shared morphological character supports the molecular evidence that these genera should be removed from the Geoglossaceae.

Asexual stages are unknown for most species in this clade, and apothecia of these fungi are most commonly found in associated with mosses (Imai, 1941; Jumpponen et al., 1997; Schumacher and Sivertsen, 1987). Species of both *Geoglossum* and *Trichoglossum* have a worldwide distribution, while *Sarcoleotia globosa* is so far mainly known from temperate areas in the Northern Hemisphere (Schumacher and Sivertsen, 1987; Spooner, 1987; Zhuang and Wang, 1998).

4.2.2.2. Ascocoryne clade (gelatinous endophyte clade). Species of three small genera *Ascocoryne*, *Neobulgaria*, and *Chloroscypha* are included in this clade, a lineage not previously recognized in the Helotiales. The presence of gelatinous tissue seems of limited use in recognizing phylogenetic relationships. Moore (1965) studied the gelatinous tissue in the Leotiomycetes and suggested four different developmental types: the coryneoid type, the cudonioid type, the leotioid type, and the bulgarioid type. *Ascocoryne* species have the coryneoid type while *Neobulgaria* and *Leotia* species have the leotioid type. The apothecia of *Chloroscypha* are only slightly gelatinous, and a gelatinous substance maybe also excreted from the paraphyses (Dennis, 1968; Petrini, 1982; Seaver, 1931, 1951). Baral (1987) studied the ring-like amyloid structures of ascus apices using light microscopic techniques and suggested that species of *Chloroscypha*, *Neobulgaria*, and perhaps *Ascocoryne* have an ascus apparatus similar to species of Sclerotiniaceae.

Apothecia of *Chloroscypha* species can be induced in vitro from the foliage of host plants, but ascospores collected from the apothecia fail to develop after germination (Petrini, 1982). This indicates that after successfully colonizing the host tissue and establishing the endophytic lifestyle, species of *Chloroscypha* may be capable of completing their life cycles as saprobes. *Ascocoryne sarcoides* has been considered to be protective against decay fungi as an endophyte and is found more frequently in roots than in stems (Basham, 1973; Whitney, 1995; Whitney et al., 2002). Fungi in this clade have a worldwide distribution.

4.2.2.3. Dermea clade (bark endophyte clade). Three species of *Dermea* and *Neofabraea* in the Dermateaceae are included in this clade. The Dermateaceae is a large, poorly

studied, and heterogeneous family (Pfister and Kimbrough, 2001; Raitviir and Spooner, 1994). Previous studies based on the ITS region suggest that our Dermea clade may also including species of *Pezicula*, *Ocellaria*, *Dermea*, and *Neofabraea* (Abeln et al., 2000; De Jong et al., 2001; Goodwin, 2002; Verkley, 1999). The only other member of the Dermateaceae we sampled, *Mollisia*, is not in this clade.

Morphologically, species of the Dermea clade produce erumpent or superficial, fleshy and small apothecia on plants, with an excipulum consisting of rounded cells with often dark walls. The hymenium in several genera in the Dermateaceae are covered by an 'epithecium', a gelatinized structure composed of tips of the paraphyses and extracellular material (Verkley, 1999). Many species of the Dermea clade produce two types of conidia, i.e., macro and microconidia, and both anamorphic and teleomorphic stages can be observed on the same stroma. Most species of *Pezicula* are pioneers that colonize twigs and branches just before they die back, typically while they remain held off the ground (Verkley, 1999). Such species are probably endophytes living in inner bark. Mature ascospores in *Pezicula* usually are septate and thick walled and embedded in the gelatinized epithecium, and they could be transferred and dispersed via feeding activities of insects or in the insects gut. Insects are well-known vectors of fungal pathogens (Saikkonen et al., 1998; Vega and Blackwell, 2005), and there are several lineages of endosymbionts in beetles' guts having independent origins in pathogenic ascomycetes (Suh et al., 2001). Data from *Pezicula* show that some species have a narrow host range, and some are even only known from a single host species (Taylor, 1983; Verkley, 1999), and this again raises the issue about vectors, particularly insects. There are some plant pathogens as well in this family, for instance, *Diplocarpon rosae*, which causes a very serious rose black-spot disease. Although poorly studied from the Southern Hemisphere, genera in this clade are world wide in distribution. At least six species of *Pezicula* or *Neofabraea* occur in New Zealand, and most of these are undescribed and some are known only from culture from studies of plant endophytes (P.R. Johnston and S. Joshee, unpublished data).

4.2.2.4. Hemiphacidium clade (gymnosperm leaf endophyte clade). The genera *Heyderia* and *Chlorencoelia* and three genera in the Hemiphacidiaceae, *Fabrella*, *Hemiphacidium*, and *Meria* (anamorph of *Rhabdocline*, Gernandt et al., 1997) are included in this clade. The Hemiphacidiaceae, proposed by Korf (1962), has been thought to be a small family in the Helotiales, but our results suggest it may need expanding to include more genera previously placed in the Helotiaceae. Stone and Gernandt (2005) proposed *Sarcotrochila* as the valid name for *Hemiphacidium*, but they were undecided about the limits of the family Hemiphacidiaceae sensu Korf, so we retain the traditional names to limit confusion.

All members of the Hemiphacidiaceae sensu Korf produce small, simple apothecia beneath the surface of leaves, and the apothecia are erumpent and push the covering host tissue back as a small scale (Korf, 1962). The ectal excipulum in these apothecia is highly reduced. In contrast, *Heyderia abietis* and *Chlorencoelia versiformis* form large, well-developed apothecia. Species in the Hemiphacidiaceae are plant pathogens or endophytes, and typically cause needle-blight or needle-cast disease. Species of *Heyderia* and *Chlorencoelia* have been regarded as saprobes, and *H. abietis* has been thought of as a decomposer of spruce needles in Europe. Endophytic stages of two *Heyderia* species have been discovered recently using molecular markers (Jean Bérubé, per. comm.). *Chlorencoelia* species can be found from wood of conifers and rotting wood of *Quercus* and *Salix* (Dennis, 1968) and they are also common on *Nothofagus* in New Zealand (http://www.landcareresearch.co.nz). If our results reflect true evolutionary relationships among these fungi, then this suggests a correlation between morphology and biology: i.e., a highly reduced apothecium is associated with a parasitic and endophytic lifestyles as in *Hemiphacidium* species, while the larger and fully developed apothecium is associated with a saprobic lifestyle as in *Chlorencoelia* species. A similar adaptation has been reported in the Rhytismatales; pathogens such as *Rhytisma* and *Lophodermium* produce simple and small apothecia on host tissues, while saprobes such as *Spathularia* and *Cudonia* produce large and complex apothecia on duff (Wang et al., 2002).

4.2.2.5. Hymenoscyphus clade (ericoid root-endophyte—aquatic saprobe clade). The genera *Cudoniella*, *Ombrophila*, and *Hymenoscyphus* included here do not always form a monophyletic group (they were weakly supported as a clade in Wang et al., 2005), and the genus *Hymenoscyphus* and similar taxa form a morphological group without obvious unifying characters (regarded as a "wastebasket" by Korf, 1973). Diverse ericoid mycorrhizal fungi have been found to be closely related to *Hymenoscyphus* based on rDNA sequences (Egger and Sigler, 1993; Monreal et al., 1999), but this relationship was not supported by recent studies using ITS (Vrålstad et al., 2002a,b; Zhang and Zhuang, 2004).

Although morphologically simple, these fungi are among the most common helotialean taxa in the field which have been found on various substrates. Species of *Cudoniella*, *Ombrophila*, and many species of *Hymenoscyphus* produce apothecia on submerged woody substrates or decaying wood in boggy places (Abdullah et al., 1981; Dennis, 1968; Descals et al., 1984; Fisher and Spooner, 1987; Fisher and Webster, 1983; Webster et al., 1995). Members of the *H. ericae* aggregate form both ecto- and ericoid mycorrhizal symbioses, and have diverse ecological attributes.

Some aquatic hyphomycetes have been documented as root endophytes (Sati and Belwal, 2005). Our study provides evidence that root endophytes, saprobic teleomorphs, and aquatic teleomorphs form a clade. However, our confidence about this relationship is not strong due to the poorly resolved phylogeny. The anamorphs of fungi in this group have been well documented for aquatic species. Various

forms of conidia have been recorded from *Cudoniella* and *Hymenoscyphus* species, often stressed as evidence for the poor correlation between the classifications of teleomorphs and anamorphs (Abdullah et al., 1981; Descals et al., 1984; Fisher and Spooner, 1987; Fisher and Webster, 1983; Marvanova, 1997; Webster et al., 1995).

4.2.2.6. Lachnum clade (hairy endophyte- saprobe clade). Two *Lachnum* and one *Bryoglossum* species appear in this clade. *Lachnum* is a large genus in the Hyaloscyphaceae, which is probably polyphyletic. Abeln et al. (2000) recognized 2 clades of hairy discomycetes, Lachnoideae (equivalent to our Lachnum clade) and Hyaloscyphoideae. Data from other studies (Cantrell and Hanlin, 1997; Wang et al., 2005) suggested that several other genera, such as *Hyaloscypha*, *Trichopezizella*, *Neodasyscypha*, *Trichopeziza*, *Solenopezia*, *Perrotia*, *Proliferodiscus*, and *Lachnellula*, belong to this clade, along with *Lachnum* and *Bryoglossum*. There are no genera in our study that represent the Hyaloscyphoideae clade of Abeln et al. (2000).

Morphologically, fungi in this group are diverse but they are all characterized by various hairs as cellular extensions from the ectal excipulum of the apothecium. Subgroups or tribes have been suggested within the family based on characters of the hairs, excipulum, paraphyses, and asci. Placing *Bryoglossum gracile* in the Geoglossaceae due to its club-shaped apothecia is artificial, since hairs are also present on the stalk of this fungus (Kankainen, 1969; Wang et al., 2005). These fungi occur on various substrates and have been treated as saprobes (e.g., Huhtinen, 1990), but this finding may need reassessment in many cases, especially in taxa with a high degree of substrate specificity. For example, *Bryoglossum gracile* is moss-inhabiting (Redhead, 1977), while some *Lachnellula* and *Lachnum* species are known to be pathogens on conifers, or are consistently associated with diseased ferns (Spooner, 1987). The life histories of these fungi are barely known, and various conidia, including *Phialophora*-like conidia, have been reported in *Hyaloscypha* and allied genera (Huhtinen, 1990). The distribution of hairy helotialean fungi is worldwide, but no collections from the Southern Hemisphere or the tropical regions were included in this study.

4.2.2.7. Leotia-Bulgaria clade (wood and litter decomposer clade). Species of *Bulgaria*, *Holwaya*, *Microglossum*, and *Leotia* form a clade in the narrower-range analyses. *Microglossum* species have been included in the Geoglossaceae along with *Geoglossum* and *Trichoglossum* primarily based on morphology, and this placement has been supported by ultrastructural studies of the ascus apex (Verkley, 1994). Close relationships between *Leotia* and *Microglossum* have been suggested by previous studies based on rDNA or protein-coding gene sequences (e.g., Gernandt et al., 2001; Landvik, 1996; Liu and Hall, 2004). Analyses based on LSU rDNA data also place *Thuemenidium* in this clade (Z. Wang, unpublished data), a genus traditionally placed in the Geoglossaceae on the basis of morphology.

The fungi in this clade are morphologically very diverse. Gelatinous structures are present in both *Leotia* and *Bulgaria* but they are classified as different types based on anatomy (Moore, 1965). Species of *Microglossum*, *Thuemenidium*, and *Holwaya* produce long, multiseptate, and hyaline ascospores. Characters of ascospores in *B. inquinans* link this fungus to the Sordariales (Döring and Triebel, 1998), which probably is the sister group of the Leotiomycetes. The biology of these fungi is barely known. In the Northern Hemisphere, *Bulgaria inquinans* is frequently collected on bark of hardwoods in the Fagaceae, and it may be a weak plant pathogen (Itzerott, 1967 cited by Döring and Triebel, 1998 therein), while *Holwaya mucida* is mostly found on wood and bark of *Tilia* (Korf, 1973). Species of *Leotia*, *Microglossum*, and *Thuemenidium* are found usually on humus rich ground, sometimes on decaying wood, but rarely on leaf litter. There are no reports of *Holwaya* from the Southern Hemisphere, but the genera *Leotia*, *Microglossum*, *Thuemenidium*, and *Bulgaria* are globally distributed. One New Zealand collection of *Microglossum* is placed outside of the clade including northern collections of *Microglossum* and *Leotia*, which suggests a long isolation period from other *Microglossum* species.

4.2.2.8. Mitrula clade (leaf parasite-aquatic saprobe clade). Three small genera, *Mitrula*, *Gremmeniella*, and *Hydrocina* are included in this clade without strong bootstrap support. This relationship has not been discovered in previous studies. The position of *Mitrula* in the Helotiales has been controversial (Eriksson, 2005; Kirk et al., 2001; Wang et al., 2005).

Hydrocina chaetocladia and *Gremmeniella abietina* both produce tiny disc-shaped apothecia with a cream-white hymenium. The receptacle of *H. chaetocladia* is colorless, with the stalk embedded in a gelatinous substance, while the receptacle of *G. abietina* is heavily pigmented and sclerotized (Puninthalingam and Gibson, 1973; Webster et al., 1991). The apothecia of *Mitrula* species are club-shaped with a bright yellow, pinkish-yellow to beige hymenium and have a reduced receptacle. Species of *Hydrocina* and *Mitrula* are known as aero-aquatic saprobes, i.e., they live on submerged substrates but produce apothecia above water level (Redhead, 1977; Wang et al., 2005; Webster et al., 1991). *Gremmeniella abietina* is known as a pathogen of conifers, and causes serious diseases especially to seedlings of pines. *G. abietina* grows also on artificial media (Petrini et al., 1989), implying that this fungus is capable of living as a saprobe. The biology of *Mitrula* species is still somewhat unclear. Conidia have been induced in vitro and may be adapted to environments such as slow moving water and vernal forest pools (Wang et al., 2005). *Hydrocina chaetocladia* produces two types of conidia, of which the macroconidia (*Tricladium chaetocladium*) are adapted to an aquatic environment. *G. abietina* causes Scleroderris-disease and produces conidia within a dark-colored stromatic pycnidium. These conidia are able to infect young shoots to start an initial infection (Gremmen, 1968, 1972).

Discharge of both conidia and ascospores in *G. abietina* requires the presence of free water (Skilling, 1969). Fungi in this clade are only known from temperate areas in the Northern Hemisphere.

4.2.2.9. Sclerotinia clade (stromatic pathogen-saprobe clade). Fungi in this clade have been well investigated in previous studies using different rDNA regions (Holst-Jensen et al., 1997a,b, 1998, 1999; Schumacher and Holst-Jensen, 1997), and several pathogenic species are amongst the best studied in the Helotiales (e.g., Dennis, 1968; Dumont, 1971; Dumont and Korf, 1971; Holst-Jensen and Schumacher, 1994; Kohn, 1979, 1982; Kohn and Schumacher, 1984; Korf, 1973; Novak and Kohn, 1991; Spooner, 1987; Zhuang, 1998).

Holst-Jensen et al. (1997b) recognized two closely related stromatic (stroma producing) groups, viz. Sclerotiniaceae (sclerotial stromata) and Rutstroemiaceae (substratal stromata), and this relationship is confirmed in this study. Holst-Jensen et al. (1997b) suggested that *Piceomphale bulgarioides* should be excluded from the Rutstroemiaceae, but our results suggest a basal position of this spruce endophyte in this clade. Sister relationships between this clade and the Hemiphacidium clade, mostly conifer endophytes with highly reduced apothecia, are strongly supported for the first time. The wider host range in the Sclerotinia clade compared to species in the Hemiphacidium clade suggests that major lineages in the Sclerotinia clade have shifted or expanded from conifer hosts to angiospermous hosts. Except for a few well-known pathogens, the lifestyles of most fungi in this clade are unknown, and they have been described as necrotrophs, opportunistic parasites, saprotrophs, and endophytes. A study of a chestnut pathogen *Sclerotinia pseudotuberosa* (= *Ciboria batschiana*) showed that the fungus occurred asymptomatically in different tissues of the host, and the endophytic behavior may represent a adaptive strategy of the pathogen for rapid and massive host colonization in favorable situations (Vettraino et al., 2005). Representatives of the Rutstroemiaceae are worldwide in distribution, whereas the Sclerotiniaceae may be primarily a northern temperate group.

4.2.2.10. Vibrissea-Loramyces clade (dark septate root endophyte-aquatic saprobe clade). Aero-aquatic *Vibrissea*, *Chlorovibrissea*, aquatic *Loramyces*, dark septate endophyte *Phialocephala fortinii*, and the wood inhabiting *Mollisia* are included in this clade. Gernandt et al. (2001) and Wilson et al. (2004) also used molecular evidence to link fungi isolated as root endophytes with aquatic fungal teleomorphs. The family Vibrisseaceae, including *Vibrissea* and *Chlorovibrissea*, is not monophyletic. Based on previous studies using data of ITS and or SSU rDNA sequences, two other endophytic genera, *Acephala* and *Rhexocercosporidium*, and one plant pathogenic *Tapesia* species, also belong in this clade (Goodwin, 2002; Grünig and Sieber, 2005; Shoemaker et al., 2002; Wilson et al., 2004).

Species of *Chlorovibrissea*, typically found on submerged or partly submerged wood in streams, and aquatic species of *Vibrissea* are morphologically similar, except that the former ones are green and probably restricted to the Southern Hemisphere (Kohn, 1989; Korf, 1990). Some species of *Vibrissea* are not aquatic and produce smaller and sessile apothecia on various substrates with a brown, sclerotium-like base (Iturriaga, 1997). A similar sclerotium-like base in *Mollisia* places this large, problematic, and probably polyphyletic genus in the Dermateaceae (Dennis, 1968; Korf, 1973). The morphology of *Loramyces* species is unique and highly adapted to an aquatic environment. Dark cells present at the base of the *Loramyces* apothecium and the hyphal structure of the apothecia are analogous to those of the Dermateaceae (Digby and Goos, 1987). Dark septate endophytes, *Phialocephala* species, are characterized by dark-colored and septate hyphae, and are associated with various plant hosts (Grünig et al., 2002). Most genera in this clade include some species, which produce conidia putatively adapted to an aquatic lifestyle. At least one *Mollisia* species has an aquatic anamorph, producing *Helicodendron* macroconidia (strongly coiled conidia designed to capture air for floating) along with *Phialophora*-like microconidia (Fisher and Webster, 1983). Two types of conidia are produced in *Vibrissea flavovirens* as well (Hamad and Webster, 1988). Fungi in this clade may have a worldwide distribution, except for species of *Loramyces* (in the Northern Hemisphere) and *Chlorovibrissea*. *Vibrissea albofusca* from New Zealand and a *Chlorovibrissea* species form a weakly supported clade outside of the clade that includes two Northern Hemisphere collections of *Vibrissea*. Convergent evolution in aquatic environments rather than geographic isolation would be the best explanation for the distant relationships within the Vibrisseaceae.

5. Conclusions

Studies of symbiotic relationships between fungi and higher plants have focused mainly on mycorrhizae, plant pathogens or endophytes and their host plants (Saikkonen et al., 1998; Allen et al., 2003). How these relationships affect the evolution of higher fungi and the diversity of woody plant endophytes, especially higher ascomycetes, has not received much attention. Analyses of data from three rDNA regions with a wide taxonomic sampling in this study improves our understanding of evolutionary relationships within the Helotiales, and provide a framework for future phylogenetic studies of this group. Our study suggests that lifestyle and ecological factors are critical in shaping the evolutionary history of the helotialean fungi. Plant endophytism is a widespread strategy used by members of the Leotiomycetes. Transformations among endophytes, parasites, and saprobes, and shifts between terrestrial and aquatic habitats may be important factors driving the high morphological diversity observed

in this group of fungi. However, more data from the rDNA regions analyzed here as well as protein-coding genes, and wider sampling from all families recognized in the Helotiales and the Leotiomycetes are required to generate a robust phylogenetic classification and to estimate the ancestral lifestyles of the Helotiales and related fungi. In addition, molecular data from environmental samples, such as plant leaves and roots, insects, soil, and water are needed for a more comprehensive view of the ecology and evolutionary relationships within the Helotiales.

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