Phylogenetic placement of four genera within the Leotiomycetes (Ascomycota)

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Abstract: Phylogenetic relationships are currently unknown for many taxa of discomycetes. Type species of three genera of Leotiomycetes (Graddonia, Propolis, and Strossmayeria) and a representative of Vibrissea were sequenced for the 28S nuclear ribosomal large subunit (LSU) to determine their phylogenetic affinities. A phylogeny of the Leotiomycetes, including numerous helotiallean taxa, was constructed under maximum likelihood and Bayesian inference. All four genera occurred in the Leotiomycetes. Graddonia occurred as an unsupported sister clade to the aero-aquatic genera Lambertella and Spirosphaera. Propolis formed a strongly supported clade with Cyclaneusma, Marthamyces, Melittosporium, and Naemacyclus as an early-diverging member of the Leotiomycetes, while the placement of Strossmayeria and Vibrissea was supported in the Vibrissea-Loramyces clade.

Key words: Ascomycota, Graddonia, Leotiomycetes, phylogenetics, Propolis, Strossmayeria, systematics, Vibrissea.

Introduction: The Leotiomycetes (Eriksson and Winka 1997), colloquially referred to as “discomycetes”, is comprised of five orders, 19 families, 641 genera, and 5587 species (Kirk et al. 


2008). The class contains the polyphyletic order Helotiiales, hereafter referred to as helotialean fungi, and is characterized by small, often brightly colored apothecial ascomata, small, thin-walled, inoperculate asci, and forcibly discharged ascospores. These fungi are most commonly encountered as saprobes and plant parasites, and may also exist as endophytes of a variety of plants (Read et al. 2000, Wilson et al. 2004, Wang et al. 2006a).

Several discomycetes with previously unknown phylogenetic affinities were encountered during a survey of terrestrial discomycetes in the Great Smoky Mountains National Park (GSMNP) during Summer and Fall 2009 as part of the All Taxa Biodiversity Inventory (ATBI) sponsored by Discover Life in America (DLIA). Over a period of 21 collecting days ranging from May to October 2009, more than 350 collections of terrestrial discomycetes representing 66 unique species and 47 new park records were made (Miller and Hustad, unpub. data). Included in these collections are representatives of four genera of helotialean discomycetes (Graddonia coracina (Bres.) Dennis, Propolis versicolor Fr., Strossmayeria basitricha (Sacc.) Dennis, and Vibrissea filispora f. filispora (Bon.) Korf & Sánchez) for which molecular sequence data is lacking and phylogenetic relationships are unknown. Furthermore, Graddonia coracina, Propolis versicolor, and Strossmayeria basitricha represent the type species of their respective genera, underscoring the need for reliable sequence data and accurate phylogenies for making informed taxonomic decisions. The LSU nrDNA region was chosen for this study because LSU sequences representing a broad range of Leotiomycetes species are available in GenBank and previous studies have shown this gene to be useful in determining phylogenetic relationships above the species level among helotialean fungi (Wang et al. 2005, 2006a, b, Raja et al. 2008). Small subunit (SSU) nrDNA is too highly conserved to resolve lineages within the Leotiomycetes (Gernandt et al. 2001), while the internal transcribed spacer (ITS) region is too variable for determining relationships above the species level due to alignment problems (Wang et al. 2006a).

The purpose of this study was to: 1) determine the phylogenetic placement of four genera of Leotiomycetes using LSU, and 2) attempt to further elucidate the phylogenetic structure within the class through the addition of a diverse array of taxa.

Materials and Methods

Taxon sampling
Discomycete ascomata were collected from woody and vegetative substrata from several sites throughout the GSMNP (Tennessee and North Carolina, USA) and documented following standard procedures (Rossman et al. 1998, Lodge et al. 2004). Voucher specimens were deposited in the fungus herbarium at the Illinois Natural History Survey (ILLS). In addition to these collections, several discomycetes received through European collaborators were also used for this study (Table 1). Specimens were identified based on the morphology of ascomata using the pertinent literature (e.g. Phillips 1887, Seaver 1928, 1951, Dennis 1960, Sánchez 1966, Korf 1973, Dennis 1978, Breitenbach and Kranzlin 1984, Gminder 1993, Hansen and Knudsen 2000). Ascomata were hand-sectioned and squash-mounted in water and images of micromorphological structures were captured with a QImaging QColor 3 digital camera mounted on either a Leica MZ7.5 dissecting microscope with a Schott KL1500 fiber optics light source or an Olympus BX51 compound microscope using differential interference microscopy. Images were processed using Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View, California). A minimum of 30 measurements was taken for all morphological structures when possible using NIH Image 1.63 (National Institute of Health, Bethesda, Maryland).
A diverse sample of Leotiomycetes taxa representing many of the sequences published by Wang et al. (2006a, b), Spatafora et al. (2006), Raja et al. (2008), and Lantz et al. (2011) were obtained from GenBank. *Neolecta vitellina* (Bres.) Korf & J.K. Rogers was chosen to root the trees based on its basal position in the Ascomycota (Landvik et al. 2001, Liu and Hall 2004). Outgroup taxa include members of the Eurotiales, Geoglossomycetes, Orbiliales, and Pezizales.

Generation of molecular data

Total genomic DNA was extracted from ascomata using a QIAGEN DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) following methods outlined in Promputtha and Miller (2010). Total genomic DNA was observed on a 1% TBE agarose gel stained with ethidium-bromide.

Partial LSU nrDNA was PCR amplified using Ready-To-Go™ PCR Beads (GE Healthcare, Waukesha, Wisconsin) containing 1-5 µL genomic DNA, 2.5 µL of 50% DMSO (dimethyl sulfoxide, Fisher Scientific, Pittsburgh, Pennsylvania) in DI water, and/or 2.5 µL of BSA (bovine serum albumin, New England Biolabs, Ipswich, Massachusetts), 1 µL each of 10 µM primer, and enough DI water to bring the reaction volume to 25 µL. Primers JS1 (Landvik 1996) and LR6 (Vilgalys and Hester 1990) were used to amplify the LSU, except if introns were present in which case LROR (Rehner and Samuels 1995) was substituted for JS1. The LSU gene was amplified using the following thermocycling parameters: initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 30s, 48°C for 15s, and 72°C for 1 min with a final extension step of 72°C for 10 min. PCR products were purified with ExoSAP-IT® (Affymetrix, Cleveland, Ohio) following manufacturer’s recommendations. Purified PCR products were used in 11 µL sequencing reactions with BigDye® Terminators v 3.1 (Applied Biosystems, Foster City, California) in combination with the following primers: JS1 or LROR, LR3, LR3R, and LR6 (Vilgalys and Hester 1990, Rehner and Samuels 1995, Landvik 1996). Sequences were generated on an Applied Biosystems 3730XL high-throughput DNA capillary sequencer at the Keck Biotechnology Center at the University of Illinois.

Sequence alignment and phylogenetic analyses

Each sequence fragment was subjected to an individual BLAST search to verify its identity. Sequences from GenBank were assembled and aligned with newly obtained sequences using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, Michigan), optimized by eye and manually corrected when necessary. Ambiguously aligned regions were excluded and then reincluded as unequivocally coded characters subjected to stepmatrices derived from pairwise comparisons of sequences (Lutzoni et al. 2000).

Unambiguously aligned characters were subjected to individual symmetric stepmatrices, which account for transition-transversion variation, using STMatrix ver. 2.2 (Francois Lutzoni and Stefan Zoller, Biology Department, Duke University). Maximum parsimony (MP) analysis was conducted using Paup*4.0b10 (Swofford 2003) as follows: constant characters were excluded, gaps were treated as a fifth character, 1000 random-addition replicates were implemented with TBR branch-swapping, MULTREES option was in effect, and zero-length branches were collapsed.

The Akaike Information Criterion (AIC) (Posada and Buckley 2004) as implemented in ModelTest 3.7 (Posada and Crandall 1998) was used to determine the best fit model of evolution (GTR+I+G) for both maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood analyses were performed using PhyML (Guindon and Gascuel 2003) under the GTR substitution model with six rate classes and invariable sites optimized. A BioNJ starting tree was constructed and the best of nearest neighbor
interchange (NNI) and subtree pruning and regrafting (SPR) tree improvement was implemented. Bootstrap support (Felsenstein 1985) was determined with 100 bootstrap replicates. Clades with ≥70% bootstrap support were considered significant and highly supported (Hillis and Bull 1993).

Bayesian analysis employing a Markov Chain Monte Carlo (MCMC) algorithm was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as an additional means of assessing branch support. The GTR+I+G model with six rate categories was implemented and four chains were run for 10 000 000 generations with trees sampled every 1000th generation resulting in 10 000 total trees. The first 1000 trees, which extend beyond the burn in phase in each analysis, were discarded and the remaining 9000 trees were used to calculate posterior probabilities using the sumt function in MrBayes 3.1.2. Clades with posterior probability ≥95% were considered significant and highly supported.

Results: The entire LSU dataset consisted of 114 total taxa including outgroups. The final LSU alignment was 942 base pairs including 372 constant, 137 uninformative, and 366 parsimony informative characters. In addition, seven ambiguous regions consisting of 67 characters were delimited and recoded for parsimony analysis. Ambiguous regions were excluded from ML and BI analyses.

The single most likely tree generated by the ML analysis is shown in Figure 1. This tree topology is identical to one of two most parsimonious trees (length=2648, CI=0.2919, RI=0.6058) generated in an unequally weighted MP analysis (the second tree differed only at unsupported nodes at the tips of the tree), as well as the consensus tree produced under BI (data not shown). All four genera were placed in the Leotiomycetes. *Graddonia coracina* formed a separate clade with two aero-aquatic genera (*Lambertella* and *Spirosphaera*) as sister taxa. *Propolis versicolor* was placed in a clade including *Cyclaneusma*, *Marthamyces*, and *Naemacyclus* and was most closely associated with *Propolis farinosa* and *Melittosporium versicolor*. *Strossmayeria basitricha* and *Vibrissea filisporia f. filisporia* occurred in the *Vibrissea-Loramyces* clade.

Discussion

Leotiomycetes

The Leotiomycetes occurred as a single monophyletic group in this analysis, but without Bayesian posterior probability and ML bootstrap support. In agreement with Wang et al. (2006b), the Helotiales is polyphyletic and several families within this group were observed to be non-monophyletic including the Helotiaceae, *Hyaloscyphaceae*, and *Vibrisseaceae*. Further molecular analysis with increased taxon sampling within these groups is needed to elucidate their taxonomic positions. Within the outgroup taxa the Eurotiales, Geoglossomycetes, Orbiliales, and Pezizales formed well-supported clades distinct from the Leotiomycetes.

Graddonia

*Graddonia* is a monotypic genus for the species *Graddonia coracina*. Originally described from Italy as *Patinella coracina* by Bresadola (1897), Dennis (1955) later established the genus *Graddonia* to contain *G. coracina*. Despite recent study of the genus (Gminder 1993), no molecular analysis has included *G. coracina* to date. Characteristics of *G. coracina* are hyaline ascospores becoming 1-septate and packed with many small oil drops in the fresh state, 8-spored inamyloid asci, as well as saprobic habitat occurring on typically submerged or very damp twigs and logs (Fig. 2).

Specimens of *G. coracina* used in this study from France, Sweden, and USA were also sequenced for ITS (data not shown), which revealed less than 1% sequence divergence, suggesting the presence of only a single Northern Hemisphere species. Our analyses support the inclusion of *Graddonia* in the Leotiomycetes where it forms a
clade with two aero-aquatic genera *Lambertella* and the anamorph *Spirosphaera*, which occur as

Figure 1: Phylogram generated from a maximum likelihood analysis of 114 ascomycete 28S nuclear ribosomal large subunit (LSU) sequences (ln L = -13579.39). Taxa in bold represent new sequences of Leotiomycetes discussed in this study. Thickened branches indicate Bayesian posterior probabilities ≥95%. Numbers above branches refer to maximum likelihood bootstrap values ≥70%. Tree is rooted with *Neolecta vitellina*, with members of the Eurotiales, Geoglossomycetes, Orbiliales, and Pezizales included as outgroup taxa.
within the Leotiomycetes. *Spirosphaera* is an aquatic anamorphic member of the Leotiomycetes characterized by aero-aquatic conidia produced under water which then float to the water surface for dispersal (Hennebert 1968). The anamorph of *Lambertella tubulosa*, *Helicodendron tubulosum* (Riess) Linder, possesses conidia that serve a similar aero-aquatic function (Glen-Bott 1955). The semi-aquatic life cycles provide a link between these species and while the anamorphic state of *Graddonia* is yet unknown, the presence of aero-aquatic conidia would lend morphological support to the observed molecular grouping of this clade.

**Propolis**

*Propolis versicolor* is a commonly encountered and conspicuous discomycete (Fig. 3) found throughout eastern North American forests on twigs from a variety of host species including *Acer*, *Corylus*, *Fagus*, *Fraxinus*, and *Quercus* (Dennis 1978).

*Propolis* was originally established by Fries (1822) as a subgenus of *Stictis* and has been the subject of much taxonomic debate (see Sherwood 1977, Dennis 1982, Holm et al. 1986, Gams 1992, Minter 2003), but is currently an accepted genus with *Propolis farinosa* as the type species. *Propolis versicolor* has been synonymized under *P. farinosa* (Minter 2003), however, our results suggest that two genetically distinct taxa exist.

Our North American collection of *P. versicolor* occurred on a long branch distant from *P. farinosa* from New Zealand that was included in analyses by Lantz et al. (2011). The LSU sequence divergence among these two sequences was 3.2%, significant enough to suggest that these species are not the same. It should be noted, however, that since *P. farinosa* and *P. versicolor* were originally described from Europe, applying European names to these disparate collections may be erroneous.
Although the placement of Propolis in the Rhytismataceae had been suggested by Johnston (1986), Spooner (1990), and Kirk et al. (2008) based on morphological data, Lantz et al. (2011) showed the genus did not belong within the core clade of Rhytismatales and similarly the genus did not group with members of this family in our analyses. As in the results presented by Lantz et al. (2011), Propolis formed a strongly supported clade (the Propolis Clade) with Cyclaneusma, Marthamyces, Melittosporium, and Naemacyclus. Due to the lack of support for a strong association with any family, we concur with Lantz et al. (2011) that the placement of the genus within the Leotiomycetes remains unclear due to poor taxon sampling.

*Strossmayeria*

*Strossmayeria* (Fig. 4) is a well studied genus of discomycete (Iturriaga and Korf 1990) that was described by Schulzer (1881) to account for saprophytic ascomycetous fungi on decorticated wood with minute, sessile apothecia, amyloid...
ascospores, and association with the dematiaceous anamorph *Pseudospiropes* Ellis. *Strossmayeria basitricha* (Sacc.) Dennis is the type species of the genus (Dennis 1960). Although the placement of *Strossmayeria* within the Helotiaceae based on morphology has been advocated by several previous authors (Ituriaga and Korf 1990, Kirk et al. 2008), our phylogeny places *Strossmayeria* within the Vibrissea-Loramyces clade of the Leotiomycetes, most closely related to *Mollisia cinerea*. This relationship is also supported by morphology: both *Strossmayeria* and *Vibrissea* produce a distinct amyloid reaction in the ectal excipulum when stained with Melzer’s reagent. The Vibrissea-Loramyces Clade is comprised of three families, Vibrisseaceae, Loramycetaceae, and members of the Dermateaceae (*Mollisia* sp.) (Wang et al. 2006b), which are quite dissimilar morphologically. Additional taxon sampling within the Vibrissea-Loramyces clade is certainly required to better understand phylogenetic relationships in this group.

**Vibrissea**

*Vibrissea filisporia* f. *filisporia* was described by Bonorden (1853) as *Sarea filisporia*. A series of name changes and synonomies resulted in this taxon being given the name *Apostemium guernesacii* (Croun & Croun) Boud. The section *Apostemium* of the genus *Peziza* was raised to genus rank by Karsten (1870) with *A. fiscellum* (Karsten) Karsten (= *Vibrissea filisporia* f. *fiscella* (Karsten) Sánchez) as type species. While some contemporary authors (Dennis 1978) consider *Apostemium* as a separate genus, others (Sánchez and Korf 1966) have suggested the proper placement of *Apostemium* as a section of the genus *Vibrissea* and synonymized *A. guernesacii* with *V. filisporia* f. *filisporia*. Although both *Apostemium* and *Vibrissea* possess long threadlike ascospores (Fig. 5D) and habitat on decomposing submerged wood, the section *Apostemium* can be distinguished from other sections of *Vibrissea* by sessile or stipitate apothecia.

![Figure 5: Vibrissea filisporia f. filisporia ANM2064. A: Apothecium, B: Mature ascus, C: Hymenium, D: Discharged ascospore, E: Paraphysis with branched apex. A=50X magnification, B-E=800X magnification.](image)

Our analyses agree with Sánchez and Korf (1966) with the placement of *Vibrissea filisporia* f. *filisporia* within the Vibrissea-Loramyces clade (*sensu* Wang et al. 2006a) with >95% Bayesian posterior probability. Furthermore, *V. filisporia* f. *filisporia* occurred as a sister taxon to *V. flavovirens* with high Bayesian posterior probability. Though molecular analysis of the type of *Apostemium, V. filisporia* f. *fiscella*, is needed to confirm the placement of *Apostemium*
as a section of Vibrissea, our results using a putative closely related taxon support this conclusion.

**Other Taxa**

In addition to the aforementioned taxa, LSU data from several other taxa encountered during this study were also included in these analyses. Two *Cudoniella* Sacc. taxa from Spain and one from North America were included in the phylogeny and their placement within the Leotiomycetes agrees with previous molecular studies (Wang et al. 2006a, b). Four outgroup taxa, *Jafnea semitosta* (Berk. & M.A. Curtis) Korf, *Microstoma floccosum* (Schwein.) Raitv., *Otidea grandis* (Pers.) Rehm, and *Wolfina aurantiopsis* (Ellis) Seaver, representing members of the operculate Pezizales were also included. The well-supported placement of these taxa within the Pezizales corroborates previous findings by Perry et al. (2007).

**Conclusions**

*Graddonia* occurred as an unsupported sister clade to taxa with various, dissimilar morphologies suggesting that additional taxon sampling is needed to better understand generic relationships within the Leotiomycetes. The separation of *Propolis versicolor* from *P. farinosa* casts doubt on the synonymy of these taxa, though sequences of material from the type localities in Europe have not yet been analyzed. The placement of *Strossmayeria* within the Leotiomycetes is reaffirmed using molecular techniques and the position of this genus within the Vibrissea-Loramycetes clade is suggested though not strongly supported due to poor taxon sampling within this group. Our findings support previous research suggesting the synonymy of *Apostemium* with *Vibrissea*, however, molecular data from the type species of the former genus, *Apostemium fiscellum* (= *Vibrissea filispora f. fiscella*), is lacking. This research will serve to provide molecular sequence and locality information for previously unsampled taxa in the Leotiomycetes and should help illuminate the poorly known evolutionary relationships within this class. Our molecular studies suggest that applying European names to taxa collected in other areas of the world may be appropriate in some cases (e.g. *Graddonia*), but should be cautioned in other situations (e.g. *Propolis*). The Leotiomycetes are grossly underrepresented in GenBank with LSU sequences available for fewer than 500 of the 5587 (<10%) known species of Leotiomycetes fungi (searched 20 May 2011). Increased taxon sampling and molecular sequencing efforts are needed to establish phylogenetic relationships and assist in the creation of a stable classification within the Leotiomycetes.

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**Literature cited**


Malpighia 11: 241-325.


Phillips, W. 1887. A manual of the British discomycetes with descriptions of all the species of fungi hitherto found in Britain, included in the family and illustrations of the genera. William Clowes and Sons, London.


Table I. List of taxa, GenBank and herbarium accession numbers, collection numbers, and locality for specimens newly sequenced in this study.

<table>
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<tr>
<th>Taxon Name</th>
<th>GenBank #</th>
<th>ILLS Herbarium #</th>
<th>Collection #</th>
<th>Locality</th>
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<td>Cudoniella clavus</td>
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<td>ILLS60488</td>
<td>ANM2087</td>
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<td>Cosby, GSMNP, Tennessee</td>
</tr>
</tbody>
</table>

Collectors names are abbreviated as follows: ANM = Andrew N. Miller, ARV = Alberto Román Vargas, S-ÅH = Sven-Åke Hanson, JF = Jacques Fournier, VPH = Vincent P. Hustad.