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Phylogenetic comparison of protein-coding versus ribosomal RNA-coding sequence data: A case study of the Lecanoromycetes (Ascomycota)

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Abstract

The resolving power and statistical support provided by two protein-coding (*RPB*1 and *RPB*2) and three ribosomal RNA-coding (nucSSU, nucLSU, and mitSSU) genes individually and in various combinations were investigated based on maximum likelihood bootstrap analyses on lichen-forming fungi from the class Lecanoromycetes (Ascomycota). Our results indicate that the optimal loci (single and combined) to use for molecular systematics of lichen-forming Ascomycota are protein-coding genes (*RPB*1 and *RPB*2). *RPB*1 and *RPB*2 genes individually were phylogenetically more efficient than all two- and three-locus combinations of ribosomal loci. The 3rd codon position of each of these two loci provided the most characters in support of phylogenetic relationships within the Lecanoromycetes. Of the three ribosomal loci we used in this study, mitSSU contributed the most to phylogenetic analyses when combined with *RPB*1 and *RPB*2. Except for the mitSSU, ribosomal genes were the most difficult to recover because they often contain many introns, resulting in PCR bias toward numerous and intronless co-extracted contaminant fungi (mainly Dothideomycetes, Chaetothyriomycetes, and Sordariomycetes in the Ascomycota, and members of the Basidiomycota), which inhabit lichen thalli. Maximum likelihood analysis on the combined five-locus data set for 82 members of the Lecanoromycetes provided a well resolved and well supported tree compared to existing phylogenies. We confirmed the monophyly of three recognized subclasses in the Lecanoromycetes, the Acarosporomycetidae, Ostropomycetidae, and Lecanoromycetideae; the latter delimited as monophyletic for the first time, with the exclusion of the family Umbilicariaceae and *Hypocenomyce scalaris*. The genus *Candelariella* (formerly in the Candelariaceae, currently a member of the Lecanoraceae) represents the first evolutionary split within the Lecanoromycetes, before the divergence of the Acarosporomycetidae. This study provides a foundation necessary to guide the selection of loci for future multilocus phylogenetic studies on lichen-forming and allied ascomycetes.

Keywords: Bayesian analyses; Bootstrap support; Lecanoromycetes; Lichen contaminants; Lichen-forming fungi; Maximum likelihood analyses; mitSSU; nucLSU; nucSSU; Protein-coding genes; Ribosomal RNA-coding genes; *RPB*1; *RPB*2; Systematics

1. Introduction

Ribosomal RNA genes are the most commonly used loci in molecular systematic studies of fungi (Lutzoni et al., 2004). Although the limited resolving power of nuclear small subunit (nucSSU), nuclear large subunit (nucLSU) and mitochondrial small subunit (mitSSU) genes is fairly well known throughout the Ascomycota, the majority of fungal phylogenies are based on one or two of these loci (Lutzoni et al., 2004). Among multilocus fungal phylogenies using RNA polymerase II genes published recently (Cheney et al., 2001; Diezmann et al., 2004; Froslev et al., 2005; Matheny, 2005; Tanabe et al., 2004, 2006), only four have used the RNA polymerase II largest subunit (*RPB*1) and/or RNA polymerase II second largest subunit (*RPB*2) to infer phylogenetic relationships among

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lichenized and non-lichenized Ascomycota (James et al., 2006: *RPB*1 and *RPB*2 [partials] in combination with nucSSU, nucLSU, nuclear ribosomal 5.8S gene [5.8S] and elongation factor 1 alpha [*EF* 1-α]; Liu and Hall, 2004: the entire DNA-dependent RNA polymerase II second largest subunit [*RPB*2] gene; Lutzoni et al., 2004: *RPB*2 [partial] in different combinations with nucSSU, nucLSU and mitSSU; Reeb et al., 2004: *RPB*2 [partial] in combination with nucSSU and nucLSU). Three of the four multi-locus phylogenetic studies that included protein-coding genes for a broad taxon sampling across the Ascomycota (Liu and Hall, 2004; Lutzoni et al., 2004; Reeb et al., 2004) have shown that *RPB*2 used alone or in combination with ribosomal data recovered multiple deep relationships within the Ascomycota that were never previously revealed or were revealed with only low support values in prior studies. Diezmann et al. (2004) compared the resolving power and support provided by ribosomal RNA-coding genes versus protein-coding genes for *Candida* (Saccharomycotina, Ascomycota) and related taxa. They found that *RPB*1 and *RPB*2 were the best phylogenetic markers. James et al. (2006) evaluated the contribution of protein data (*RPB*1, *RPB*2, and *EF*1-α) and ribosomal data (nucSSU, nucLSU, and 5.8S) for basal relationships within Fungi. None of these genes, when analyzed individually, provided strong support for basal internodes that were highly supported in the combined six-locus phylogeny and they detected significant conflict among selected gene partitions, including *RPB*1 and *RPB*2 used individually or in combination versus ribosomal data used in combination. However, these conflicts did not decrease statistical support obtained in the combined six-locus analyses. In another recent study focusing on inoperculate Pezizomycotina, Lumbsch et al. (2005) compared the resolving power of four ribosomal genes (nucSSU, nucLSU, mitSSU, and mitochondrial large subunit [mitLSU]) used individually or in combinations, and evaluated the utility of mitLSU for resolving deep phylogenetic relationships within the lichenized ascomycetes. They concluded that the backbone of the euascomycetes phylogeny remained poorly resolved with the addition of partial sequences from the mitLSU to the three other ribosomal RNA-coding loci.

The collaborative project Assembling the Fungal Tree of Life (AFTOL), provided an opportunity to investigate the contribution of five loci (nucSSU, nucLSU, mitSSU, *RPB*1, and *RPB*2), individually and in combinations, to phylogenetic resolution and statistical confidence, across a large number of species sampled in the Lecanoromycetes (Eriksson, 2006), which includes 90% of all described lichen-forming species and represents the largest class of fungi. The *RPB*1 locus is used here for the first time in a phylogenetic study of the Lecanoromycetes and this is the first study on lichen-forming fungi based on five loci. We address difficulties inherent to a selective amplification of the mycobiont for these loci. To perform an adequate comparison among loci, we sampled, when possible, comparable amount of nucleotide data from each gene (1.4 kb of nucSSU, 1.4 kb of nucLSU, 0.8 kb of mitSSU, 1.0 kb of *RBP*1 and 0.7–1.2 kb of *RPB*2). The resolving power and contribution toward phylogenetic confidence of each locus separately and in various combinations was assessed at different taxonomic levels, ranging from closely related species to subclass level relationships, by comparing support values derived from maximum likelihood bootstrap analyses on each data set and by comparing the distribution of unequivocal transformational changes (under the maximum parsimony optimization criterion) on the combined five-locus phylogeny. Finding the optimal combinations of genes for two-, three-, and four-locus data sets with the greatest level of resolution and support was the main goal of this study.

2. Materials and methods

2.1. Taxon sampling and molecular techniques

For this study, we sampled 100 taxa: 10 outgroup species from the Leotiomycetes including Geoglossaceae (8 species) and the Lichinomycetes (2 species) following James et al. (2006) and Spatafora et al. (2006); and 90 ingroup species representing three recognized subclasses in the Lecanoromycetes, the Acarosporomycetidae (4 species, 1 family from the Acarosporales), the Ostropomycetidae (13 species, 1 family from the Agyriales, 2 families from the Ostropales, 2 families from the Pertusariales, and Hymeneliaceae), the Lecanoromycetidae (68 species, 12 families from the Lecanorales, 5 families from the Peltigerales, 3 families from the Teloschistales, and the Umbilicariaceae) and 2 taxa with unknown placement (Eriksson, 2006), *Lopezaria versicolor* (Lecanoromycetes) and *Phyllobaeis erythrella*, Baeomycetaceae (Ascomycota) (Supplement 1). DNA was isolated from freshly collected lichen thalli except for *Acarosporina macrospora* (from culture). A standard DNA isolation procedure employing 2% SDS lysis buffer (Zolan and Pukkila, 1986) was used. Isolated DNA was resuspended in sterile water and stored at −20 °C. When pigments or polysaccharides inhibited PCR, the DNA isolates were cleaned using the E.Z.N.A.® Fungal DNA Miniprep Kit (Omega Biotech). PCR amplification followed a modified Vilgalys and Hester (1990) procedure using 1.5–3.0 mM $MgCl_2$, 0.4 mg μl^{-1} of bovine serum albumin (Hillis et al., 1996), Red Hot® DNA Polymerase and chemistries from ABgene® (ABgene Inc., Rochester, New York, USA). Cloning, when required, was performed with a TOPO TA Cloning® Kit (Invitrogen™, life technologies, Carlsbad, CA, USA). Amplified PCR products were purified with the QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA) prior to automated sequencing using Big dye chemistry with 3700 or 3730xl DNA analyzers (PE Applied Biosystems, Foster City, CA, USA).

We amplified and sequenced the five following loci: 0.8 kb of mitSSU using primers mitSSU1–mitSSU3R (Zoller et al., 1999), 1.4 kb of nucSSU using primers 131F (or NS1)-NS22 (or NS24) (White et al., 1990), 1.4 kb nucLSU

Table 1
Primers designed to amplify RNA polymerase II (*RPB*1 and *RPB*2) for members of the Ascomycota [asc], Lecanoromycetidae [lecan] and Peltigerales [pelt]

*RPB*1	
*RPB*1	
(A–D region[a], 1.2 kb):	
*RPB*1-AFasc:	5′-ADTGYCCYGGYCATTTYGGT-3′
*RPB*1-AFlecan:	5′-TGYCCYGGYCATTTYGGTGT YAT-3′
*RPB*1-AFpelt:	5′-TGYCCYGGYCATTTYGGTC AYAT-3′
*RPB*1-6Rlasc:	5′-ATGACCCATCATRGAYTCCT TRTG-3′
*RPB*1-6R2asc:	5′-ATGACCCATCATRGAYTCCT-3′
*RPB*1	
(D–G[b] region[a], 2.1 kb):	
*RPB*1-DF2asc:	5′-CAYAAGGARTCYATGATGGG TCAT-3′
*RPB*1-DF2asc:	5′-CAYAAGGARTCYATGATGG-3′
*RPB*1-GlRasc:	5′-ACNCCNACCATYTCNCCNGG-3′
*RPB*1FR-seq1[c]	5′-TANCCNGTYTCNGCNGTYTT-3′
*RPB*1FR-seq2	5′-CGYTGRATRTANCCNGTYTC-3′
*RPB*2	
*RPB*2 (5–7[b] region[a])	
*RPB*2-5Fpelt:	5′-TTCAACAARCTBACVAARGA TGT-3′
*RPB*2 (7–11 region[a])	
*RPB*2-7Fpelt:	5′-GAAGAAACBGTVATGATTG TSATGAC-3′

More information about these new primers can be found at http://www.lutzonilab.net/primers.

[a] Position and size of *RPB*1 and *RPB*2 conserved domains can be found at http://faculty.Washington.edu/benhall/.

[b] These regions of RNA polymerase II were not used in this study but were amplified and sequenced for other AFTOL-linked studies (James et al., 2006; Miadlikowska et al., 2006).

[c] These primers have been designed for sequencing [seq] only.

using primers LR0R–LR7 (or LR5) (Vilgalys and Hester, 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm), 1.0 kb of *RBP*1 using primer *RPB*1-A_f (Stiller and Hall, 1997) and primers designed for this study (*RPB*1 region A–D; Table 1), and 0.7–1.2 kb of *RPB*2 using primers f*RPB*2-7cF-f*RPB*2-11cR (*RPB*2 region 7–11; Liu and Hall, 2004) and primers designed for this study (Table 1). These primers and other potentially useful primers for fungal systematics can be found at http://www.aftol.org/data.php and http://www.lutzonilab.net/primers/. PCR and sequencing conditions can be found in Hofstetter et al. (2002). Sequences were assembled and edited using the software package Sequencher™ 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Alignments of the nucSSU, nucLSU, mitSSU, *RPB*1 (A–F), and *RPB*2 (7–11) sequences for the 100 taxa listed in Supplement 1 were prepared using PAUP* and MacClade 4.05 (Maddison and Maddison, 2002). Ribosomal genes were aligned based on the secondary structure of *Saccharomyces cerevisiae* (Kjer, 1995) provided by Cannone et al. (2002). All alignments are available at the AFTOL website (http://www.aftol.org/data.php) and http://www.lutzonilab.net/publications.

2.2. Phylogenetic analyses

In this study, multiple sequences for a given locus were often recovered from a single lichen thallus. To separate sequences of targeted mycobionts from contaminants, we verified the identity of each sequence by blasting against GenBank data and by conducting preliminary phylogenetic analyses. To determine the phylogenetic affiliation of contaminants, we prepared a data matrix for nucSSU consisting of 349 representatives of the Ascomycota with an emphasis on lichens, endolichenic fungi (fungi living asymptomatically within lichen thalli), and endophytic fungi (Arnold et al., 2007). We added sequences of recovered contaminants to this data set and performed Neighbor-Joining bootstrap analyses (NJ-bs) on 1000 bootstrap replicates, with distance measure estimated by maximum likelihood (ML) under a six-parameter (GTR, Rodriguez et al., 1990) 'best-fit' evolutionary model for nucleotide substitution (Cunningham et al., 1998; Lio and Goldman, 1998; Yang et al., 1994) using PAUP* 4.0b10 (Swofford, 2002). 'Best-fit' evolutionary models were estimated for all analyses using hierarchical likelihood ratio tests (LRTs) as implemented in Modeltest v. 3.06 (Posada and Crandall, 1998).

Topological incongruence among our data sets was examined using 1000 replicates of ML bootstrapping (ML-bs) with the GTRMIX model and gamma distribution conducted in RAxML-VI-HPC (Stamatakis et al., 2005) on each locus separately and on all possible combinations (26) of the five loci. To screen for putative conflict, we used the program compat.py (available at www.lutzonilab.net), which compared ML-bs values for all possible pairwise combinations of the five loci. A conflict was assumed to be significant if two different relationships (one being monophyletic and the other being non-monophyletic) for the same set of taxa were both supported with bootstrap values $\geqslant 70\%$ (Mason-Gamer and Kellog, 1996). Based on this criterion, eight conflicting taxa were excluded from further analyses (see Supplement 1). The final data for the five loci used in further analyses included 92 taxa.

A maximum likelihood search for the most likely tree on the five-locus data set for 92 congruent taxa was completed on 500 replicates using RAxML with the same settings as applied in the bootstrap analyses. An ML search was also conducted on the five-locus data set with *RPB*1/3rd and *RPB*2/3rd codon position excluded. In addition, bayesian analyses using Bayesian Metropolis coupled Markov chain Monte Carlo algorithm (B-MCMCMC) as implemented in MrBayes v3.1.1 (Huelsenbeck and Ronquist, 2001) were completed on the five-locus data set including nine partitions (nucSSU, nucLSU, mitSSU, *RPB*1/1st, 2nd, 3rd and *RPB*2/1st, 2nd, 3rd). Bayesian analyses were implemented with four independent chains, with every 500th trees sampled for 5,000,000 generations, using a GTR model of

nucleotide substitution, with an estimated proportion of invariable sites and a gamma distribution of four categories. To ensure that all runs converged to the same log-likelihood stationary level, we conducted three independent B-MCMCMC runs.

Phylogenetic support for the combined five-locus data set was derived from Bayesian posterior probabilities (PP) with a majority-rule consensus tree built from the last 4000 trees of each run (a total of 12,000 trees) and bootstrap values obtained from 1000 replicates of ML bootstrapping conducted with RAxML. Bayesian posterior probabilities $\geqslant 95\%$ and ML-bs $\geqslant 70\%$ were considered to be significant. Support for all other possible combinations of loci was estimated on 1000 bootstrap replicates conducted with RAxML.

Distribution of unequivocal transformational changes for nine partitions of the five-locus data set (nucSSU, nucLSU, mitSSU, *RPB*1/1st, 2nd, 3rd and *RPB*2/1st, 2nd and 3rd) on the most likely tree derived from the ML analysis (Fig. 1) was obtained using MacClade 4.05 Trace All Changes option from the Trace menu under maximum parsimony optimization criterion. Changes of nucleotides were weighted according to a symmetric step matrix calculated using the program STMatrix 2.2 (written by S. Zoller as outlined in Miadlikowska et al., 2002).

3. Results

3.1. Data sampling and lichen contaminants

Nearly all PCR products, except mitSSU, required cloning because of the presence of multiple or residual bands or because chromatograms obtained by direct sequencing of single PCR products involved multiple peaks at certain positions. PCR was performed on 4–16 clones and the resulting products selected for sequencing. BLAST and phylogenetic analyses (see Section 2) allowed us to distinguish lichen mycobiont sequences from non-lichenized contaminants.

NucSSU and nucLSU contaminant sequences that were recovered by amplification of DNA isolated from lichen thalli are listed in Table 2. We identified a total of 59 sequences of non-lichenized fungal contaminants obtained from 26 lichen species and up to eight different sequences were recovered from a single thallus (*Echinoplaca strigulacea*, AFTOL 106). Fungal contaminants represented three fungal phyla: Ascomycota (48), Basidiomycota (10), and Zygomycota (1). Basidiomycota contaminant sequences were most similar to Heterobasidiomycetes (Tremellaceae [4] and Sebacinaceae [1]) and Homobasidiomycetes representing four orders: Stereales (Stereaceae [1] and Atheliaceae [1]), Agaricales (Agaricaceae [1]), Aphyllophorales (Corticiaceae [1]), and Thelephorales (Thelephoraceae [1]). Ascomycota contaminants were from four classes/subclasses within the Pezizomycotina: the Dothideomycetes (nine sequences representing at least five orders and five families), the Chaetothyriomycetes (nine sequences, with seven of them being most similar to Herpotrichiellaceae), the Sordariomycetes (Magnaporthaceae of inc. sed. [3], Hypocreales [2] and Xylariales [1]) and the Leotiomycetes (six sequences mainly from the Helotiales [4], Cyttariales [1], and Thelebolales [1]). Six sequences were most similar to Dothideales/Chaetothyriales of inc. sed. and 12 sequences represented non-classified mitosporic Ascomycota.

Bootstrap support recovered from NJ bootstrap analyses on the nucSSU data set indicated that ten lichen contaminants (sequences most similar to Magnaporthaceae [3], Chaetothyriomycetes/Chaetothyriomycetidae [4] and Dothideomycetes/Chaetothyriomycetes of inc. sed. [3]) were nested within a clade representing non-lichenized Chaetothyriomycetidae species, i.e., *Capronia* and related genera (NJ-bs = 65%). Five of the six sequences blasting on *Lecophagus muscicola* (Table 2) and recovered from thalli of six different lichen genera belonging to the Lecanorales and Peltigerales were clustered together as a monophyletic group (NJ-bs = 99%), but with unresolved placement within the Ascomycota. Four other nucSSU contaminant sequences from the Lecanorales and Peltigerales were also monophyletic (NJ-bs = 100%) and were nested within the '*Saccharomyces*' clade (NJ-bs = 72%). Phylogenetic affiliation of the remaining contaminant sequences remains unknown within the Ascomycota.

3.2. Alignments and five-locus data set

Our data consisted of 460 sequences, of which 175 sequences were newly generated for this study (nucSSU: 31, nucLSU: 32, mitSSU: 37, *RPB*1: 40, *RPB*2: 35; Supplement 1) and most of the remaining 285 sequences (GenBank) resulted from other AFTOL projects (Geiser et al., 2006; James et al., 2006; Lutzoni et al., 2004; Miadlikowska et al., 2006; Spatafora et al., 2006). A summary of alignment lengths and number of included sites for each locus for 92 taxa (Supplement 1) is shown in Table 3. Each of the three ribosomal genes (nucSSU, nucLSU, and mitSSU) provided only a small proportion (17–29%) of unambiguously aligned characters compared to more than 80% of unambiguously aligned characters derived from the protein-coding genes (*RPB*1 and *RPB*2) included in phylogenetic analyses. Ribosomal sequences contained many insertions (40) whereas each of the RNA polymerase II genes hosted only one spliceosomal intron. In the combined five-locus data set (nucSSU+nucLSU+mitSSU+*RPB*1+*RPB*2), 4862 sites were unambiguously aligned and included in phylogenetic analyses. This data set provided 1702 putative parsimony informative characters (IC) of which 60% came from the *RPB*1 and *RPB*2 genes, with the greatest number of characters derived from the 3rd codon position (Table 3).

3.3. Combined five-locus phylogeny for the Lecanoromycetes

Our combined five-locus maximum likelihood phylogeny is well resolved and well supported, including deep

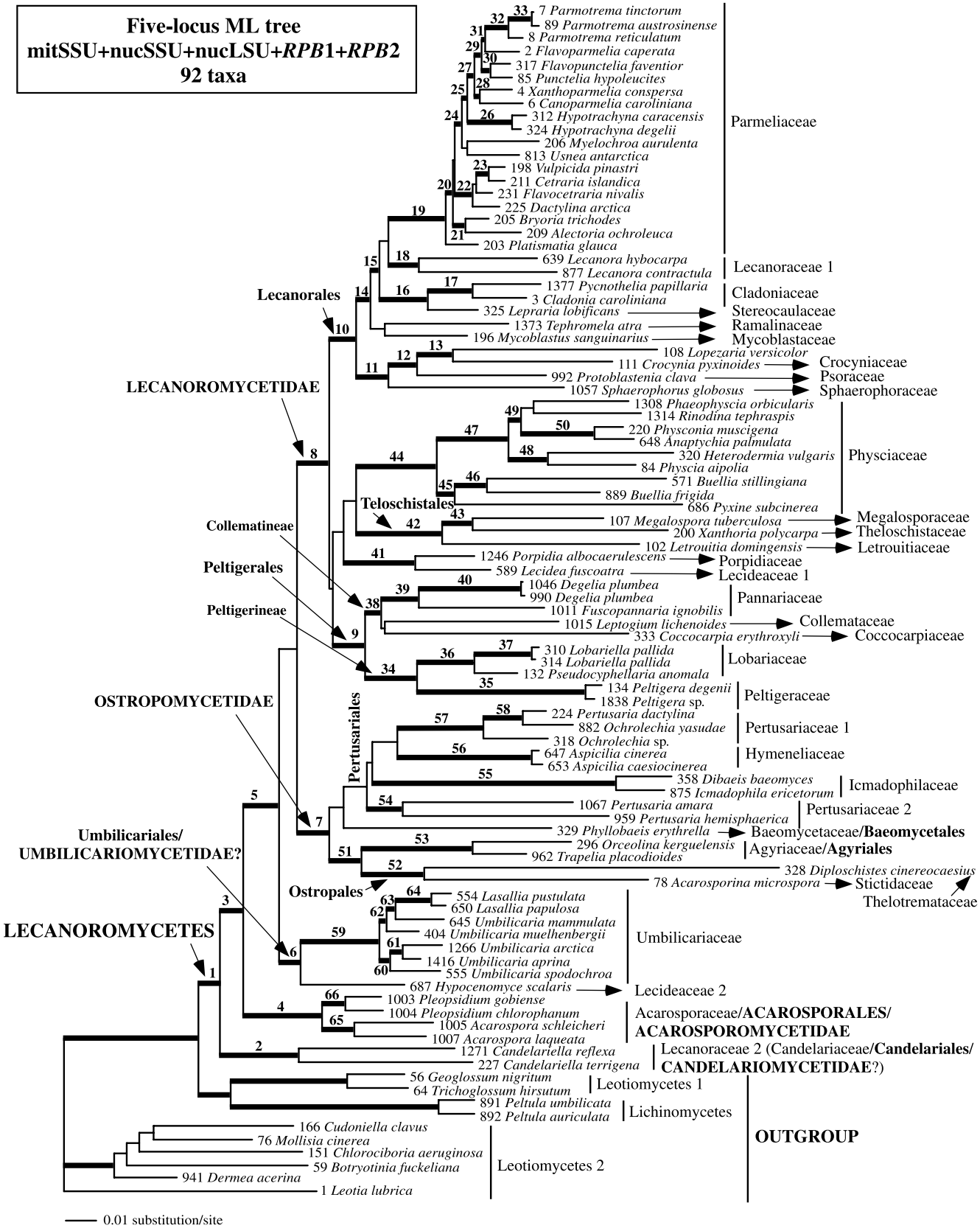

Fig. 1. Phylogenetic relationships among 82 members of the Lecanoromycetes using ten outgroup species based on maximum likelihood analysis of a combined five-locus data set (nucSSU, nucLSU, mitSSU, *RPB*1, and *RPB*2; ln likelihood = −94269.304948). Thick internodes received ML bootstrap support ⩾70%. All significantly supported internodes within the Lecanoromycetes are numbered (1–66). ML-bs support values derived from each locus separately and from all combinations of the five loci were compared for the 66 numbered internodes and are presented in Fig. 2. Taxonomical names above the subordinal level are shown in bold. Classification follows Eriksson (2006).

Table 2
Host lichens and their respective fungal contaminant sequences detected in this study

Host lichen	GenBank Accession No.		BLAST top score record (December 2005)
	nucSSU	nucLSU	
Lecanoromycetes			
Lecanoromycetidae			
Lecanorales			
Catillariaceae			
213 *Toninia sedifolia*[a]	EF053553		Sordariomycetes incertae sedis; mitosporic Magnaporthaceae
Lecideaceae			
687 *Hypocenomyce scalaris*	EF053554		Dothideomycetes; Pleosporales; Sporormiaceae
Parmeliaceae			
211 *Cetraria islandica*	EF053555		Dothideomycetes; mitosporic Dothideales
2 *Flavoparmelia caperata*	EF053556		Mitosporic Ascomycota; *Lecophagus muscicola*
89 *Parmotrema austrosinense*	EF053557		Heterobasidiomycetes; Tremellomycetidae; Tremellales; Tremellaceae
7 *Parmotrema tinctorum*	EF053558		Sordariomycetes incertae sedis; mitosporic Magnaporthaceae
203 *Platismatia glauca*		EF053596	Heterobasidiomycetes; Tremellomycetidae; Tremellales; Tremellaceae
5 *Usnea strigosa*[a]	EF053559		Dothideomycetes; Myriangiales; Myriangiaceae
	EF053560		Mitosporic Ascomycota; *Lecophagus muscicola*
198 *Vulpicida pinastri*		EF053597	Leotiomycetes; Helotiales; mitosporic Helotiaceae
		EF053598	Leotiomycetes; Helotiales; Geoglossaceae
Physciaceae			
648 *Anaptychia palmulata*	EF053561		Mitosporic Ascomycota, *Capnobotryella.* sp.
	EF053562		Dothideomycetes/Chaetothyriomycetes; incertae sedis; Tubeufiaceae
Ramalinaceae			
642 *Bacidia schweinitzii*[a]	EF053563		Dothideomycetes; unclassified Dothideomycetes
	EF053564		Dothideomycetes/Chaetothyriomycetes; mitosporic Mycosphaerellaceae
86 *Ramalina complanata*[a]	EF053565		Heterobasidiomycetes; Tremellomycetidae; Tremellales; Tremellaceae
	EF053566		Dothideomycetes/Chaetothyriomycetes incertae sedis; Dothioraceae
	EF053567		Mitosporic Ascomycota; *Lecophagus musicola.*
	EF053568		Chaetothyriomycetes; Chaetothyriales; Herpotrichiellaceae; *Capronia* sp.
	EF053569		Mitosporic Ascomycota; *Capnobotryella* sp.
Peltigerales			
Lobariaceae			
128 *Lobaria scrobiculata*	EF053570		Mitosporic Ascomycota; *Lecophagus muscicola*
132 *Pseudocyphellaria anomala*		EF053599	Homobasidiomycetes; Stereales; Stereaceae
		EF053600	Chaetothyriomycetes; Chaetothyriales; mitosporic Herpotrichiellaceae
		EF053601	Heterobasidiomycetes; Heterobasidiomycetidae; Sebacinales; Sebacinaceae
	EF053571		Sordariomycetes; Hypocreomycetidae; mitosporic Hypocreales
	EF053572		Mitosporic Ascomycota; *Lecophagus muscicola*
	EF053573		Dothideomycetes; Pleosporales; Sporormiaceae
Pannariaceae			
337 *Erioderma verruculosum*[a]		EF053602	Leotiomycetes; Helotiales; mitosporic Helotiaceae
		EF053603	Dothideomycetes; Capnodiales; mitosporic Capnodiaceae
		EF053604	Hymenomycetes; Homobasidiomycetes; Stereales; Atheliaceae
	EF053574		Chaetothyriomycetes; Chaetothyriales; Herpotrichiellaceae; *Cladophialophora* sp.
	EF053575		Sordariomycetes; Hypocreales; mitosporic Clavicipitaceae
	EF053576		Leotiomycetes; Cyttariales; Cyttariaceae
133 *Erioderma sorediatum*[a]	EF053577		Homobasidiomycetes; Aphyllophorales; Corticiaceae
	EF053578		Dothideomycetes/Chaetothyriomycetes; Dothioraceae; *Aureobasidium* sp.
	EF053579		Leotiomycetes; Thelebolales; Thelebolaceae
334 *Parmeliella* sp.[a]	EF053580		Sordariomycetes incertae sedis; mitosporic Magnaporthaceae
	EF053581		Mitosporic Ascomycota; *Leucophagus muscicola*
129 *Protopannaria pezizoides*[a]	EF053582		Ascomycota incertae sedis; mitosporic Myxotrichaceae
	EF053583		Ascomycota; mitosporic Ascomycota; *Tricladium patulum*
	EF053584		Leotiomycetes; Helotiales; mitosporic Dermateaceae
222 *Protopannaria pezizoides*[a]	EF053585		Zygomycota; Zygomycetes; Entomophthorales; Basidiobolaceae
Peltigeraceae			
134 *Peltigera degenii*	EF053586		Homobasidiomycetes; Thelephorales; Thelephoraceae
Incertae sedis			
Umbilicariaceae			
645 *Umbilicaria mammulata*	EF053587		Chaetothyriomycetes; Chaetothyriales; Chaetothyriales inc. sed.
Ostropomycetidae			
Ostropales			
Gomphillaceae			

(*continued on next page*)

Table 2 (*continued*)

Host lichen	GenBank Accession No.		BLAST top score record (December 2005)
	nucSSU	nucLSU	
106 *Echinoplaca strigulacea*[a]		EF053605	Sordariomycetes; Xylariomycetidae; Xylariales; Xylariaceae
		EF053606	Heterobasidiomycetes; Tremellomycetudae; Trenellales; Tremellaceae
		EF053607	Chaetothyriomycetes; Chaetothyriales; mitosporic Herpotrichiellaceae
		EF053608	Chaetothyriomycetes; Chaetothyriales; mitosporic Herpotrichiellaceae
		EF053609	Chaetothyriomycetes; Chaetothyriales; mitosporic Herpotrichiellaceae
		EF053610	Homobasidiomycetes; Agaricales; Agaricaceae
	EF053588		Dothideomycetes; unclassified Dothideomycetes
	EF053589		Dothideomycetes/Chaetothyriomycetes; inc. sed.; Tubeufiaceae
105 *Gyalideopsis vulgaris*[a]	EF053590		Mitosporic Ascomycota; *Coniosporium* sp.
Lichinomycetes			
Lichinales			
Lichinaceae			
896 *Lichinella iodopulchra*[a]		EF053611	Dothideomycetes; Pleosporales; Phaeosphaeriaceae
	EF053591		Mitosporic Ascomycota; *Cryomyces* sp.
	EF053592		Dothideomycetes/Chaetothyriomycetes inc. sed.; Botryosphaeriaceae
Peltulaceae			
892 *Peltula auriculata*	EF053593		Chaetothyriomycetes; Chaetothyriales inc. sed.
Incertae sedis			
108 *Lopezaria versicolor*	EF053594		Dothideomycetes; Capnodiales; Coccodiniaceae
	EF053595		Chaetothyriomycetes; Chaetothyriales; mitosporic Herpotrichiellaceae

Lichen classification follows Eriksson (2006).

[a] AFTOL lichen specimens not included in phylogenetic analyses due to missing data.

Table 3
Comparison of five loci for their potential contribution to this phylogenetic study on the Lecanoromycetes based on the 92-taxon data sets

Locus/alignment	nucSSU	nucLSU	mitSSU	*RPB*1 (A–F)	*RPB*2 (7–11)
Expected length[a] (bp)	1300	1400	800	700–1150	950
Recovered length for PCR products (bp)	1300–1950	1150–2800	700–2000	700–1200	950–1050
Alignment length (bp)	6744	4011	2625	1185	1029
Number of introns	22	12	6	1	1
Number of spliceosomal introns	10	7	0	1	1
Ambiguously aligned regions (bp/%)	5619/83	2852/71	2150/82	222/19	69/7
Non-ambiguously aligned regions (bp)	1125	1159	475	963	960
Number of parsimony informative characters (IC)	202	296	183	546	475
Number of IC per codon position				lst:142/2nd:90/3rd:314	lst:103/2nd:58/3rd:314

[a] Length estimated based on primer positions provided by http://www.lutzonilab.net/primers/ for the nucSSU and nucLSU; Zoller et al. (1999) for the mitSSU; Matheny et al. (2002), Stiller and Hall (1997) and this study (Table 1) for the *RPB*1; and Liu et al. (1999) and this study (Table 1) for the *RPB*2.

phylogenetic relationships. Of the 80 internodes reconstructed within the ingroup, 66 received ML-bs support $\geqslant$70% (Fig. 1). Bayesian analysis on the same five-locus data set (tree not shown) revealed significant posterior probability support (PP $\geqslant$95%) for 63 out of the 66 internodes significantly supported by bootstrap values (Fig. 2). In our phylogeny, all three subclasses recognized in the Lecanoromycetes, the Acarosporomycetidae, Ostropomycetidae, and Lecanoromycetidae (internodes 4, 7, and 8, respectively) are well supported as monophyletic. Two additional distinct deep lineages, the *Candelariella* group (internode 2) and Umbilicariaceae group (the family Umbilicariaceae+*Hypocenomyce scalaris*; internode 6) were reconstructed with high bootstrap support (>70%). All deep relationships among major groups within the Lecanoromycetes are well supported, except for the Lecanoromycetidae being sister to the Ostropomycetidae. The genus *Candelariella* (Lecanoraceae 2) represents the first evolutionary split in the Lecanoromycetes (internode 1) followed by the Acarosporomycetidae and a large clade containing the *Umbilicaria* group, the Ostropomycetidae and Lecanoromycetidae (internode 5). Phylogenetic relationships within the Lecanoromycetidae and Ostropomycetidae are partly supported, including the monophyletic Lecanorales (internode 10), Teloschistales (internode 42) and Peltigerales (internode 9) in the Lecanoromycetidae. With the exception of the Pertusariaceae and Lecanoraceae, all families represented by more than two genera are delimited as monophyletic (the Parmeliaceae, internode 19; Physciaceae, internode 44; and Umbilicariaceae, internode 59). Many terminal relationships (at the family and intra-family levels) received high bootstrap support with this current sampling.

3.4. Resolving power and support provided by the five loci

Based on the ML-bs analyses on each gene separately, *RPB*1 provided support for more than half (58%) of all the internodes supported in the five-locus bootstrap analysis

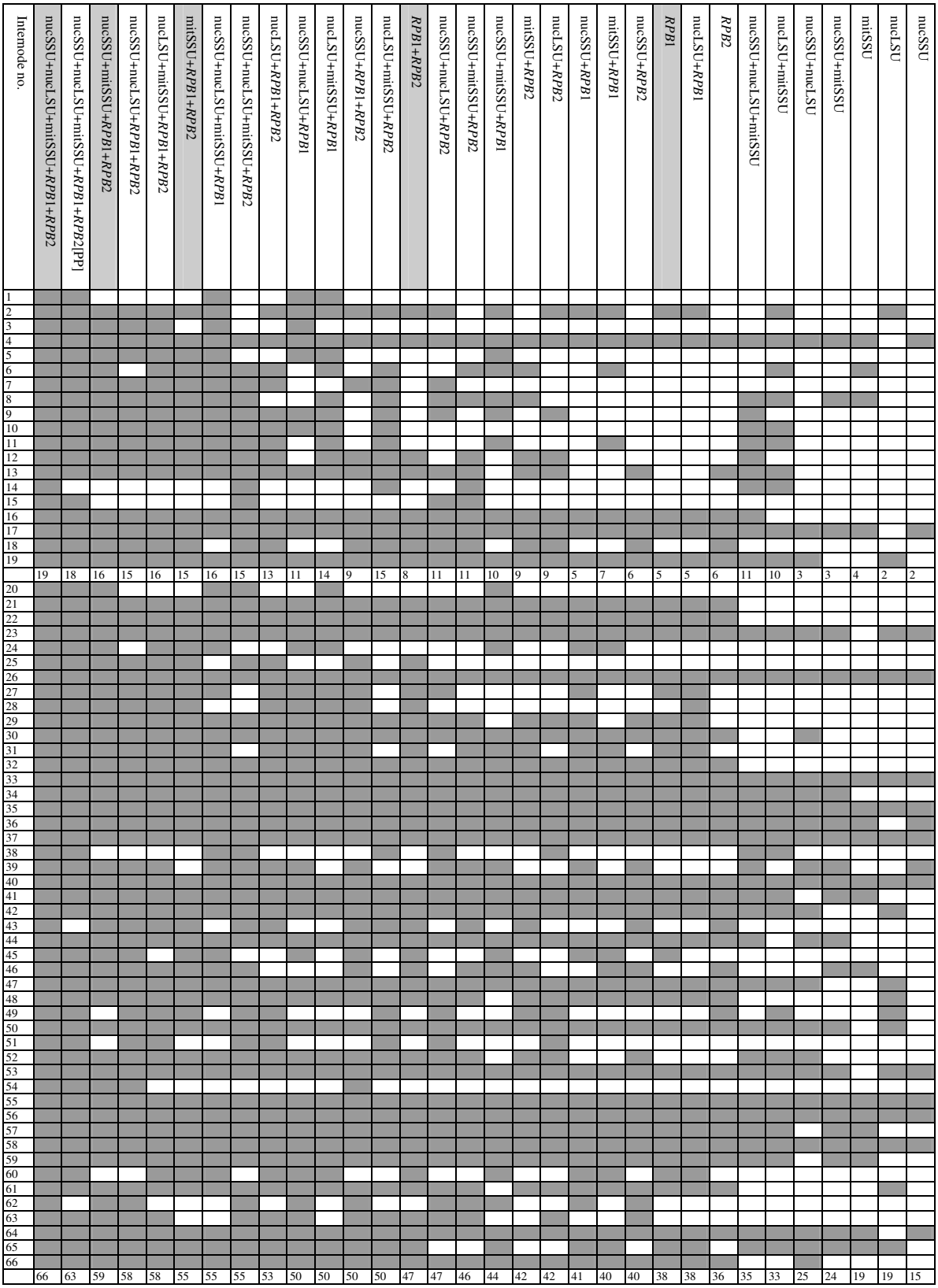

Fig. 2. Comparison of ML-bs support derived from each locus separately and from different combinations of five loci (31 data sets), and PP support derived from the combined five-locus data set for 66 internodes (second column) selected in Fig. 1. Dark gray boxes indicate significant support (ML-bs $\geqslant 70\%$ or PP $\geqslant 95\%$). Last row indicates the total number of significantly supported internodes for each data set. The row after internode 19 indicates the total number of significantly supported deep internodes in our phylogeny (internodes 1–19). Each combination of loci (four-, three-, two- and one-locus analyses) that performed the best in term of the total number of significantly supported internodes is shaded pale gray.

(Fig. 2). The *RPB*2 supported two fewer nodes than *RPB*1, followed by nucLSU and mitSSU, both supporting 29% of the total number of selected internodes. *RPB*1 and *RPB*2 genes individually were phylogenetically more efficient than all two- and three-locus combinations of ribosomal loci. Finally, nucSSU supported only 23% of the 66 internodes. Of the 38 internodes supported by *RPB*1, five were unique for this locus, and represented phylogenetic relationships mostly at the family and intra-family levels. *RPB*2 and mitSSU each supported two internodes not reconstructed from the other single gene bootstrap analyses (internodes 18 and 43 revealed by the *RPB*2 data and internodes six and eight revealed by the mitSSU data).

Among two-locus data sets, the combined two protein-coding genes (*RPB*1+*RPB*2) performed the best. The *RPB*1 and *RPB*2 combined data supported 12 more internodes than the combined three ribosomal genes (nucSSU+nucLSU+mitSSU). Only 28 of all significantly supported internodes were common to both analyses and 26 internodes were supported exclusively by one or the other combination of data (19 internodes by *RPB*1+*RPB*2 and 7 internodes by nucSSU+nucLSU+mitSSU). Most of these 19 internodes supported by the *RPB*1+*RPB*2 data set were found in terminal parts of the tree and represented internal relationships within families (e.g., internodes 19–33 in the Parmeliaceae; internodes 44, 46–47 in the Physciaceae; internodes 60–61, 63 in the Umbilicariaceae; internode 66 in the Acarosporaceae; and internode 43). The seven internodes supported by the three-locus combination of ribosomal data represented mostly deep phylogenetic relationships at the subclass, order and interfamily levels (e.g., the Lecanoromycetidae—internode 8, the Peltigerales—internode 9, and the Lecanorales—internodes 10–12). The remaining two-locus combinations with *RPB*1 or *RPB*2 were slightly worse (5–9 fewer supported internodes) than *RPB*1 with *RPB*2 together. The two-locus combinations of nucSSU with mitSSU and nucLSU provided support only for 51% and 53% of the internodes supported by the combination of protein data.

Our bootstrap analyses suggest that the best three-locus combination was a concatenation of protein data with mitSSU (mitSSU+*RPB*1+*RPB*2; 55 supported internodes). However, replacing mitSSU with nucLSU (nucLSU+*RPB*1+*RPB*2) supported only two fewer internodes. The least powerful combination of three loci was for ribosomal data (nucSSU+nucLSU+mitSSU; 35 supported internodes). All four-locus data sets performed almost equally well; however, combinations including both *RPB*1 and *RPB*2 were slightly better than the others by supporting 3–4 supplementary internodes that were not significantly supported in the combinations including ribosomal genes and *RPB*1 or *RPB*2.

In general, adding genes increased the number of supported internodes except for the *RPB*1 locus, which performed equally well when analyzed alone and in combination with the nucLSU (nucLSU+*RPB*1; Fig. 2). None of the single locus data sets performed very well in the backbone (internodes 1–19) of the five-locus phylogeny (Figs. 1 and 2—the row following internode 19). Ribosomal genes individually supported 2–4 deep internodes, whereas protein-coding loci supported only 2–3 more internodes (a total of 5–6 internodes). However, when combined, ribosomal data (nucSSU+nucLSU+mitSSU) significantly supported three more basal internodes (a total of 11 internodes) than protein-coding genes (*RPB*1+*RPB*2; a total of eight internodes). The most efficient two-locus combination to support deep phylogenetic relationships was the nucLSU+mitSSU data set (10 supported internodes). Three-locus combinations including mitSSU and nucLSU with *RPB*2 or *RPB*1 performed better than the remaining three-locus data sets (14–15 versus 9–13 supported internodes), except for the combination of mitSSU with *RPB*1 and *RPB*2 with 15 well supported internodes, and almost as well as all four-locus combinations, which provided high confidence for 15–16 of the 19 deep internodes supported in the five-locus phylogeny.

3.5. Repartition of transformational changes for each locus based on the combined five-locus phylogenetic analysis

The total number of unequivocal transformational changes reconstructed under the maximum parsimony criterion for the 66 selected internodes in the five-locus phylogeny (Fig. 1) varied from seven (internode 25) to 196 (internode 55) (Supplement 2). With only one exception, the greatest and/or the second greatest number of changes observed for the targeted internodes were derived from the *RPB*1/3rd and *RPB*2/3rd codon position (the highest number of IC; Table 3). Internode 55 (monophyly of the Icmadophilaceae) was the only one with the highest number of changes provided by ribosomal genes (nucLSU- 45 changes, and nucSSU- 34 changes); however, this internode received only one change less from the *RPB*2/3rd codon position compared to the nucSSU (33 versus 34). Among the three ribosomal genes, the nucLSU was the best in providing the first, second or third highest number of changes for 32 internodes. The nucSSU, mitSSU and *RPB*2/1st codon position performed at a similar level in obtaining the second or the third highest score for 16, 15, and 14 internodes, respectively. The least number of changes was provided by *RPB*1/1st, *RPB*2/2nd and *RPB*1/2nd codon positions (the lowest number of IC; Table 3). For several internodes only one of the three ribosomal genes provided supplementary changes to the protein-coding genes (e.g., internodes 3, 25, 32, and 41) and for two internodes no contribution of ribosomal genes was recorded (internodes 21 and 22) when considering only unequivocal changes reconstructed using maximum parsimony as the optimization criterion (Supplement 2).

4. Discussion

4.1. Lichen thalli contaminants

A high level of phylogenetic resolution and support for phylogenetic trees are the main criteria used to determine

the appropriateness of loci for molecular systematic studies. Several studies focusing on the systematics of lichen-forming Ascomycota discussed these attributes for different loci (Lumbsch et al., 2005; Lutzoni et al., 2004; Reeb et al., 2004), but none compared the effort necessary to recover nucleotide sequence data from each locus. In our study, amplification of ribosomal genes, with the exception of mitSSU, was much more difficult than for protein-coding genes because of co-amplification of contaminant fungi commonly occurring in lichen thalli (Table 2).

Numerous and taxonomically diverse fungi have been isolated in pure cultures from non-sterilized and surface sterilized lichen thalli (Arnold et al., 2007; Girlanda et al., 1997; Petrini et al., 1990). By direct cloning of PCR products we recovered sequences of three fungal phyla (Ascomycota, Basidiomycota, and Zygomycota) apart from lichen sequences. The majority of these sequences represent a broad taxonomic diversity within the Ascomycota (the Dothideomycetes, Chaetothyriales, Leotiomycetes, and Sordariomycetes). With inclusion of 6 inc. sed. contaminants, the Dothideomycetes and Chaetothyriales were the most frequent groups among lichen contaminants (44%). Because the Dothideomycetes and Sordariomycetes include a high number of fungi isolated from surface sterilized lichens (Arnold et al., 2007), some of the lichen contaminants recovered here may represent undiscovered endolichenic fungi (fungi living asymptomatically in lichen thalli). It is very likely that cloning PCR products obtained from genomic DNA derived directly from lichen thalli allowed recovery of some endolichenic fungi, which do not grow on artificial media, and therefore, have not been recorded so far. As reported by Arnold et al. (2006), these two approaches (cloning and isolation of endophytes in pure cultures) were found to be complementary in terms of capturing the diversity of endophytes from plants. Many members of the Chaetothyriales are known to be associated with lichen thalli as secondary fungi (lichenicolous fungi; Lawrey and Diederich, 2003). Interestingly, NJ bootstrap analysis of nucSSU indicated that five of the contaminant sequences were closely related to *Capronia pilosella* (NJ-bs = 64%), a genus known to include lichenicolous fungi. Another monophyletic group of five lichen contaminant sequences (NJ-bs = 99%) did not cluster with any of the other 359 Ascomycota nucSSU sequences present in the NJ bootstrap analysis. This clade includes contaminants recovered from various lichens of the Lecanorales and Peltigerales, which were collected in different parts of the USA (*Usnea strigosa* and *Flavoparmelia caperata* in North Carolina, *Ramalina complanata* in Texas, *Lecanora hybocarpa* in Tennessee, *Lobaria scrobiculata* and *Pseudocyphellaria anomala* in Oregon; Table 2). These phylogenetically closely related contaminant species exhibit high nucSSU similarity (98–99%) with *Lecophagus muscicola* (Table 2). *Lecophagus* and related genera are rotifer-catcher fungi with an unknown ecology (Tanabe et al., 1999). As rotifers feed on lichens, the widespread and frequent occurrence of *Lecophagus* (and very likely other related fungi) in lichen thalli can be beneficial for lichens by providing protection against rotifers.

As an aside, we investigated the effect of thalli sterilization and recovery of mycobiont DNA (data not shown). To avoid fungi attached to lichen surfaces, selected lichen thalli were rinsed and surface sterilized (sequential sterilization using 95% alcohol, followed by 10% bleach and 70% alcohol) before DNA isolation following Arnold et al. (2007). This approach allowed us to eliminate most lichen surface contaminants but decreased substantially the quantity of extracted DNA for lichen mycobionts. Bleach used alone at a concentration of 10–30% had the same negative effect, in reducing DNA quantity, as the complete sterilization procedure. Sterilization with alcohol (70–95%) only, did not sufficiently reduce the number of recovered non-lichen sequences.

For the nucSSU and nucLSU loci, we considered designing primers specific to certain groups within the Lecanoromycetes. This solution would be perhaps very efficient for studies focusing on selected lichen groups, especially for foliose or soil lichens, which appeared to be more contaminated by accessory fungi than saxicolous species. However, it was impossible to design mycobiont-specific primers within a class framework because the putative targeted regions in the nucSSU and nucLSU were too conserved or too variable (often CT rich). Consequently cloning of PCR product was chosen as the less time-consuming approach than designing primers for each lichen family.

Ribosomal genes in lichen-forming fungi include many introns and other types of insertions (Bhattacharya et al., 2000; Cubero et al., 2000; DePriest, 2004; DePriest and Been, 1992; Gargas et al., 1995; Simon et al., 2005). In several studies, PCR was reported to favor amplification of short fragments over the long ones (Quist and Chapela, 2001; Sagerström et al., 1997; Suzuki and Giovannoni, 1996). Contaminant fungi, having often fewer introns than the lichenized host, were then likely to be favored over the lichenized mycobiont during the amplification process. This bias was less pronounced for protein-coding genes, which in general contain very few introns (Table 3). Therefore, for protein-coding genes, sequencing a single clone would allow us to recover the targeted mycobiont, while for ribosomal genes (nucSSU and nucLSU), sequencing of 4–16 clones was required. This fact suggested that lichen mycobiont was the most abundant template available for PCR in our genomic DNA extractions. Nevertheless, high dilutions of DNA extracts (500–1000x) did not allow us, except in a few cases, to suppress co-amplification of contaminants.

4.2. Phylogenetic relationships within the Lecanoromycetes

Our phylogeny (Figs. 1 and 2) strongly supported and confirmed the monophyly of the Acarosporomycetidae reconstructed as the second evolutionary split within the Lecanoromycetes, and the monophyly of the Ostropomy-

cetidae. The largest subclass, the Lecanoromycetidae, is for the first time delimited as monophyletic with the exclusion of the family Umbilicariaceae+*Hypocenomyce scalaris* (internode 8; ML-bs > 70%, PP > 95%), which represents a separate lineage in the Lecanoromycetes. Because the sister relationship of the Lecanoromycetidae with the Ostropomycetidae is not supported in the five-locus phylogeny (although ML-bs = 74% based on the nucSSU+ nucLSU+ *RPB*1+*RPB*2; tree not shown), it is still possible for the Umbilicariaceae group to be sister to the Lecanoromycetidae, therefore a putative member of this subclass. Independent of future delimitation of the Lecanoromycetidae, the family Umbilicariaceae, including related genera (e.g., *Hypocenomyce*), should be recognized at the order level and perhaps at the subclass level (see Miadlikowska et al., 2006). The close relationship of the Umbilicariaceae and the genus *Hypocenomyce* (Lecideaceae) as well as other genera not included in this study (*Fuscidea*, *Maronea* [Fuscideaceae], *Elixia* [Elixiaceae], *Boreoplaca* [Lecanoromycetes genera inc. sed.], and *Ophioparma* [Ophioparmaceae]) was previously shown and discussed (Lumbsch et al., 2004; Lutzoni et al., 2004; Miadlikowska et al., 2006; Reeb et al., 2004; Wedin et al., 2005).

In agreement with Miadlikowska and Lutzoni (2004), the Lecanoromycetidae includes two well-supported main lineages—the Lecanorales and Peltigerales, and an unsupported clade containing Teloschistales, Physciaceae, Porpidiaceae and part of the Lecideaceae (Lecideaceae 1). In agreement with Eriksson's classification (Eriksson, 2006) the following families belong to the Lecanorales: the monophyletic Parmeliaceae, Cladoniaceae, Stereocaulaceae, Ramalinaceae, Mycoblastaceae, and part of the Lecanoraceae (Lecanoraceae 1). Several deep internodes within the Lecanorales, and particularly in the Parmeliaceae, are well supported, e.g., the *Parmotrema*-clade (Blanco et al., 2006) comprising the genera *Xanthoparmelia*, *Canoparmelia*, *Flavoparmelia*, *Parmotrema* (including former *Rimelia*), *Punctelia* and *Flavopunctelia*; and the cetrarioid clade (*Cetraria*, *Flavocetraria* and *Vulpicida*) including *Dactylina* (Fig. 1). *Lopezaria*, considered as a genus of uncertain position within the Lecanoromycetes (Eriksson, 2006), is shown here to be a member of the Lecanorales. Because of our limited taxon sampling (many families are represented in our phylogeny by a single genus) it is not possible to discuss the monophyly of these underrepresented taxa and their detailed phylogenetic affiliations. These relationships are discussed in a phylogenetic study of the Lecanoromycetes based on a data set of 274 taxa (Miadlikowska et al., 2006).

The order Peltigerales comprises two suborders, the Collematineae (Coccocarpiaceae, Collemataceae, and Pannariaceae) and the Peltigerineae (Lobariaceae, Nephromataceae [not sampled in this study] and Peltigeraceae), as defined by Miadlikowska and Lutzoni (2004).

Although not supported in the five-locus phylogeny, the close relationship of the family Physciaceae with the order Teloschistales (Teloschistaceae, Letrouitiaceae, and Megalosporaceae; Lutzoni et al., 2004; Reeb et al., 2004) received significant support (ML-bs = 72%) based on the nucSSU+nucLSU+mitSSU+*RPB*1 data (tree not shown) and therefore revealed its potential inclusion in this order. Phylogenetic affiliation of the Porpidiaceae+Lecideaceae 1 in the Lecanoromycetidae remains uncertain based on this taxon sampling; however, see Miadlikowska et al. (2006).

In this study (Fig. 1), the subclass Ostropomycetidae contains the order Ostropales, sister to the Agyriales, the Pertusariales (monophyly not supported) and the Baeomycetaceae, a family with an uncertain placement in the Ascomycota (Eriksson, 2006). Based on ribosomal genes, Kauff and Lutzoni (2002) proposed an elevation of the Baeomycetaceae to the order level in the Ostropomycetidae. Although our study confirmed the inclusion of the Baeomycetaceae (represented by *Phyllobaeis erythrella*) in the Ostropomycetidae, its accurate placement in the Ostropomycetidae remains uncertain. Phylogenetic relationships within the order Pertusariales represented by the polyphyletic Pertusariaceae (1 and 2), Hymeneliaceae and Icmadophilaceae are not supported in this phylogeny.

One of the most interesting and unexpected results of this study is the placement of the genus *Candelariella*, a member of the Lecanoraceae according to Eriksson (2006), outside of the Lecanorales and Lecanoromycetidae (also found and discussed by Wedin et al., 2005). However, our five-locus phylogeny indicates that this family is part of the first evolutionary split within the Lecanoromycetes (before the divergence of the Acarosporomycetidae) (Fig. 1), contrary to Wedin et al. (2005) two-locus phylogeny reporting *Candelariella* as sister to the Acarosporaceae. This led Wedin et al. (2005) to include *Candelariella* within a monophyletic group that they refer to as the *Acarosporaceae*-group. This relationship was highly supported only by Bayesian posterior probability in their study, and could be due to an artifact resulting from current implementations of Bayesian Markov chain Monte Carlo methods as described in Alfaro et al. (2003) and Lewis et al. (2005). This genus, together with *Candelaria*, was previously classified in its own family, Candelariaceae (Hakulinen, 1954), but due to its ascus type it was always considered a close relative to the Lecanoraceae. If the phylogenetic position of the Candelariaceae in the Lecanoromycetes, as reported here, is confirmed with a more extensive sampling (including the genus *Candelaria* and related genera), this lineage should be elevated to the subclass level (Candelariomycetidae).

Due to the limited taxon sampling (few or missing members from many families) included in this five-locus phylogeny, no changes are proposed to the current lichen classification of the Ascomycota (Eriksson, 2006). Changes are proposed in a parallel phylogenetic study designed specifically to investigate relationships among members of the Lecanoromycetidae (Miadlikowska et al., 2006), which is based on 274 taxa and multilocus data sets (nucSSU, nucLSU, mitSSU, *RPB*1 and *RPB*2) using a supermatrix approach.

4.3. Choosing loci for phylogenetic studies of the Lecanoromycetidae

Our main goal was to determine which loci provide the highest resolving power and statistical support, and therefore, are most appropriate for systematic studies on lichen-forming fungi. To address this question, we selected the class Lecanoromycetes where most of the lichen-forming fungi are concentrated (Eriksson, 2006).

Several previous studies showed that protein-coding genes offer high resolution and support in fungal systematics (Diezmann et al., 2004; Hirt et al., 1999; James et al., 2006; Matheny et al., 2002; Morehouse et al., 2003; Tanabe et al., 2004, 2006) particularly the *RPB*2 locus in phylogenetic studies of the Pezizomycotina (Liu and Hall, 2004; Liu et al., 1999; Lutzoni et al., 2004; Reeb et al., 2004). Our results (Fig. 2) confirmed that protein-coding loci (*RPB*1 and *RPB*2) significantly supported more internodes than RNA-coding genes (nucSSU, nucLSU and mitSSU) (Figs. 1 and 2). The loci *RPB*1 and *RPB*2 individually and combined appear to be the optimal single- and two-locus data sets to use in phylogenetic studies of lichen-forming Ascomycota. Concatenating these two protein-coding genes had the most positive enhancement compare to subsequent additions of ribosomal loci. Among the ribosomal genes, the mitSSU and nucLSU individually and in combination with protein-coding genes (three-locus data set) performed much better than the nucSSU, which is commonly used in phylogenetic studies of lichen-forming fungi and Ascomycota in general. In this study, it was the most difficult gene to amplify for members of the Lecanoromycetes. However, when combined with both *RPB*1 and *RPB*2 in four-locus data sets, all three combinations of ribosomal genes exhibited a similar level of phylogenetic efficiency (58–59 supported internodes).

Two recent studies focusing on the Lecanoromycetes (Lumbsch et al., 2004; Wedin et al., 2005) concluded that the ribosomal genes nucLSU and mitSSU are very useful in delimiting major clades within the Lecanoromycetes. In our analyses these two loci provided high support for some deep to intermediary internodes (1–19, Fig. 2), which were poorly supported in the combinations involving the *RPB*1+*RPB*2 data (mitSSU: internodes 6 [the Umbilicariales] and 8 [the Lecanoromycetidae]; nucLSU+mitSSU: internodes 10, 11, 14 [the Lecanorales and internal relationships]). Overall the combined nucLSU+mitSSU performed only slightly better in the backbone of the tree than two-locus combinations involving the *RPB*2 gene (nucLSU+mitSSU supported two more internodes than *RPB*1+*RPB*2 and one more internode than mitSSU+*RPB*2 and nucLSU+*RPB*2; Fig. 2), even if less data from protein-coding genes were used compared to the ribosomal loci (in this study 3.5 kb of ribosomal data versus 2.1 kb of protein-coding genes; Table 3). It is very likely that additional sequencing of *RPB*1 (region F–G; e.g., Miadlikowska et al., 2006) and/or *RPB*2 (region 5–7; e.g., Reeb et al., 2004) would improve the performance of these two genes, including support for deep internodes.

Our gene ranking based on the ML-bs criterion was corroborated by the repartition of unequivocal transformational changes on the most likely tree for the five-locus data set (Fig. 1 and Supplement 2). The major contributors to almost all selected internodes were the 3rd codon position characters from *RPB*1 and/or *RPB*2. Phylogenetic signal at the 3rd codon position of these two protein-coding genes was not saturated for this study within the Lecanoromycetes because phylogenetic analyses on the combined five-locus data set with the *RPB*1/3rd and *RPB*2/3rd codon position excluded, revealed a similar topology (tree not shown), but lower bootstrap support for several internodes compared with the complete five-locus phylogenetic tree. Ribosomal genes contributed to increase support values for many of the 66 internodes, however, in most cases their contribution was much lower (remarkably fewer transformational changes) than for protein-coding genes. Ribosomal genes were not helpful in resolving terminal internodes (e.g., no transformational changes in the Parmeliaceae: internodes 21, 22, 25, 27, 31, 32; and low number of transformational changes in the Umbilicariaceae: internodes 60–62) in the broader context of the Lecanoromycetes where most of the fast evolving ribosomal sites are excluded as parts of ambiguously aligned portions of these genes (but see Reeb et al., 2004).

Although ribosomal data appeared to be useful in some parts of the Lecanoromycetes phylogeny, it is necessary to point out two main disadvantages of using ribosomal genes versus protein-coding genes: (1) ribosomal data, except from the mitSSU, are more difficult to recover for lichen mycobionts because of PCR bias toward co-extracted contaminant DNA, and (2) ribosomal data are more problematic to align because they are not assigned to a reading frame, leading to the exclusion of the most fast evolving sites that are most often associated with regions of the alignment with short (1–2 bp) indels that cannot be aligned unequivocally (Lutzoni et al., 2000).

Because of the complementary support provided by genes capable of resolving phylogenetic relationships at different systematic ranks, combining loci in general improved phylogenetic confidence. In this study, ribosomal genes supported some basal relationships within Lecanoromycetidae that were not significantly reconstructed by protein data (Fig. 2), whereas *RPB*1 and *RPB*2, used individually or in combinations, was better in providing support for terminal relationships (within Parmeliaceae, Physciaceae, and Umbilicariaceae) that remained weakly supported based on ribosomal loci. For example, by adding one more gene, the mitSSU, to the *RPB*1+*RPB*2 data, the number of supported internodes went up from 47 to 55. Although, in this study, adding data generally improved phylogenetic confidence, a few exceptions were noticed. For example, combining nucLSU with *RPB*1 data did not increase the number of significantly supported internodes than using *RPB*1 alone. Other examples include the

monophyly of the Lecanoromycetidae (Umbilicariaceae excluded) and the Ostropomycetidae supported by nucSSU+nucLSU+*RPB*1+*RPB*2 (ML-bs = 74%) and the monophyly of the Physciaceae with the Teloschistales (ML-bs = 72%) by nucSSU+nucLSU+mitSSU+*RPB*1, but these relationships, although resolved, were not supported by the five-locus data set (Fig. 1).

In conclusion, *RPB*1 and *RPB*2 should be the first choice genes for molecular phylogenetics on lichen-forming fungi. For a multilocus study, the addition of mitSSU to these two protein-coding genes is the most efficient in term of sequencing effort.

Based on this study, as well as previous studies focusing on Ascomycota or deep relationships in Fungi (James et al., 2006; Liu and Hall, 2004; Lumbsch et al., 2005; Lutzoni et al., 2004; Miadlikowska et al., 2006; Reeb et al., 2004; Rokas et al., 2003; Wedin et al., 2005), it becomes evident that more characters, protein-coding genes in particular, and more extensive taxon sampling is required to reconstruct fully resolved and robust phylogenies for the Lecanoromycetes and Fungi in general. A five-locus data set as shown here seems to be a minimum requirement for such large-scale phylogenetic studies, especially as more taxa will continue to be added and the complexity of the phylogeny will increase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.10.016.

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