

Utility of nuclear SSU and LSU rDNA data sets to discover the ordinal placement of the Coccotremataceae (Ascomycota)

H. Thorsten Lumbsch^{1,*}, Imke Schmitt¹, Maria Ines Messuti²

¹Botanisches Institut, Universität Essen, Essen, Germany

²Centro Regional Universitario Bariloche, Universidad Nacional de Comahue, Unidad Postal, Quintral 1250, Prov. Río Negro, 8400 San Carlos de Bariloche, Argentina

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Abstract

Informal ascomycete classifications have traditionally been based in part on ascomatal morphologies. The problems associated with grouping taxa using ascomatal characters are evidenced in the Coccotremataceae where the ascomata have been interpreted either as apothecia or perithecia. We used SSU rDNA sequences representing all classes of the Pezizomycotina to infer the phylogenetic position of the family. The Coccotremataceae clustered within the Lecanoromycetes. Since the Lecanoromycetes are characterized by the presence of apothecia, these data support the apothecial interpretation, given that the ascomata of the Coccotremataceae are not the result of convergent evolution. To evaluate the ordinal placement of the Coccotremataceae we used sequences of the SSU rRNA and LSU rRNA gene of 12 Lecanoromycetes. The SSU and LSU portions of this second analysis reveal conflicting phylogenies. Therefore we compared the two portions with additional statistical tests: splits decomposition, an analysis of the distribution of homoplasy, and a calculation of the ideal nucleotide substitution rate. In all of these tests the LSU data performed better than the SSU data. The results of the incongruence length difference (ILD) test suggest the data portions could be combined. There is no difference in the tree topology of the combined data set and of the LSU portion only, but the bootstrap values in the combined tree are lower. We argue that the low bootstrap supports in the combined tree are due to the phylogenetic signal in the SSU data set. Therefore we use the LSU and the combined tree to base our classification of the Coccotremataceae. In the LSU and the combined tree the inclusion of the Coccotremataceae in the Pertusariales is supported as is the sister relationship of the Pertusariales and Agryariales. Within the Pertusariales the Coccotremataceae and Pertusariaceae are well-supported sister taxa.

Key words: ascomata, Ascomycota, Coccotremataceae, lichens, LSU rDNA, SSU rDNA

Introduction

Filamentous ascomycetes are characterized by hyphal growth and the presence of ascomata. Traditionally, the ascoma-types played an important role in the classification of filamentous ascomycetes. Generally, ascomycetes were distinguished based on ascoma-types, i.e. discomycetes with apothecia having exposed hymenia, pyrenomycetes with flesh-like ostiolate perithecia and plectomycetes with cleistothecia. This classification has often been criticized as being too schematic and consequently other characters have been considered, such as ascoma development (Nann-

feldt, 1932), or ascus-structure (Luttrell, 1951). Molecular data (Berbee & Taylor, 1992, 1995; Gargas & Taylor, 1995; Liu et al., 1999; Lumbsch et al., 2000; Spatafora, 1995) seem to support at least parts of the traditional views. Contemporary views on ascomycete classification are quite complex and comprise a combination of character sets, including ascoma-type. Most of the classes currently recognized among the higher ascomycetes, Pezizomycotina sensu Eriksson & Winka (1997), are more or less uniform regarding the main ascoma-type and in some cases appear to be apomorphic characters for classes, such as perithecia in the Sordariomycetes.

*Corresponding author: PD Dr. H. Thorsten Lumbsch, Botanisches Institut, Universität Essen, Universitätsstraße 5, D- 45117 Essen, Germany, Fax ++49-201-183-4290, e-mail: lumbsch@uni-essen.de

There are cases in which fruiting bodies cannot be easily identified as belonging to one of the three types using morphological and ontogenetical characters. Consequently, such fungi were placed in different orders. Molecular data may help with the phylogenetic estimation in these cases. We have examined the small family Coccotremataceae (currently including ca. six accepted species, Lumbsch & Messuti, unpubl. results), as an example of ascomycetes, whose nature of the ascomata is still uncertain. Zahlbruckner's (1926) classification of lichenized fungi placed members currently circumscribed in the Coccotremataceae into three families. Two of these families (Phyllopyreniaceae, Pyrenulaceae) are comprised of perithecial lichens (Pyrenocarpeae) and the third (Pertusariaceae) is comprised of apothecial lichens (Gymnocarpeae). Thus members of this family were interpreted by Zahlbruckner (1926) to have either perithecia or apothecia. Brodo (1973) carefully avoided any commitment in his revision of North American *Coccotrema* spp., but compared the diverse characters of the genus with the corresponding features in pyrenocarpous and discocarpous lichenized fungi. However, he stressed that a more thorough study of the ascoma development would be necessary before any final decision could be made regarding the nature of ascomata in *Coccotrema*. Later, using ontogenetic characters, the ascomata in the Coccotremataceae were either interpreted as modified apothecia (Henssen, 1976), or perithecia (Lumbsch et al., 1994). Morphological characters obviously do not provide sufficient data to decide the classification of this family.

Indeed, the ascomata of the Coccotremataceae are peculiar. They are pyriform to globose, opening only with a small ostiolum and in some species have protuberances of the thalline margin. The ostiolum is covered with thin hyphae, similar to paraphyses in perithecia. However, paraphysis-like lateral paraphyses are also known from lichenized discomycetes (Henssen, 1995) and perithecioid apothecia are also known from ascomycete groups with apothecia, such as Gyalectales and many Pertusariales (Ott & Lumbsch, 2000). Other characters also do not provide a clear picture. Thick-walled ascospores and a chemistry characterized by the presence of β -orcinol depsidones, are interpreted as characters suggesting close affinities to the Pertusariales (e.g., by Brodo 1973). However, the structure of the exciple, the ascus structure and the presence of cephalodia are quite different from the characters observed in the Pertusariales (Brodo, 1973; Honegger, 1982).

Based on the differences in ascoma development and anatomical characters mentioned above, Henssen (1976) referred the genera *Coccotrema* and *Lepolichen* to the Coccotremataceae, but did not provide a description for the new family. The family name was later validated (David & Hawksworth, 1991) and is listed as a

family of uncertain position in the most recent outline of Ascomycota (Eriksson, 1999).

In this study molecular data were used to infer the phylogenetic position of the Coccotremataceae. First, SSU rDNA sequence data were used to roughly estimate the phylogenetic placement within the ascomycetes. Then SSU and LSU rDNA sequence data of this family and related groups were employed to examine the phylogenetic position of the Coccotremataceae more precisely. The two data sets examined revealed conflicting topologies. In order to uncover the phylogeny of the Coccotremataceae we compared the utility of the two molecular markers with various statistical tests. We assessed differences in the two data sets using different approaches, including splits decomposition and likelihood-based methods.

Materials and methods

Specimens

Ascomata and thallus material for SSU and LSU rDNA sequence analyses of 12, resp. 13 species was used as listed in Tab. 1. DNA from the same material was used for both SSU and LSU rDNA sequencing.

DNA extraction and PCR amplification

Total DNA was extracted from fresh and herbarium material using a modified CTAB method (Cubero et al., 1999).

Dilutions (10^{-1} or 10^{-10}) of the total DNA were used for PCR amplifications of the genes coding for the nuclear SSU and LSU rRNA. Primers (primer nomenclature follows Gargas and DePriest, 1996) for amplification were: nu-SSU-0021-5' (Gargas and DePriest, 1996), nu-SSU-0819-5', nu-SSU-1293-3', nu-SSU-1750-3' (Gargas & Taylor, 1992) for the nuclear SSU rRNA gene, and nu-LSU-0155-5' (Döring et al., 2000), nu-LSU-1432-3' (=LR7) and nu-LSU-1125-3' (=LR6) (Vilgalys homepage:

<http://www.botany.duke.edu/fungi/mycolab/primers.htm#>

Large subunit RNA (25-28S) primer sequences) for the nuclear LSU rRNA gene. Amplifications were performed in 25 μ L volumes containing a reaction mixture bead (Pharmacia Biotech. Inc. Ready to Go PCR kit). 2.5 μ L diluted DNA, 2.5 μ L of each primer (10 μ M), and 17.5 μ L H_2O were added. The amplification was performed in a Stratagene Robocycler using the following program: initial denaturation at 94°C for 5 min, and 40 cycles of 94 °C for 1.3 min, 48°C for 1.5 min, 72 °C for 2 min, and a 4°C soak.

Sequencing

Fragments were cleaned using the QIAquick PCR Purification kit (Qiagen) and sequenced using the AmpliTaq DNA Polymerase FS Dye Terminator Cycle Sequencing kit (Perkin Elmer). To obtain complete, overlapping sequences in both directions the following sequencing primers were used: a) for the

Table 1. Sequences used for phylogenetic analysis with Genbank accession numbers (newly obtained sequences in bold).

Species	Collection	Class	Order	GenBank - SSU	GenBank - LSU
<i>Arthonia radiata</i> (Pers.) Ach.	—	Arthoniomycetes	Arthoniales	U23537	—
<i>Capronia mansonii</i> (Schol-Schwarz) E. Müll., Petrini, P.J. Fisher, Samuels & Rossman	—	Chaetothryomycetes	Chaetothryiales	X79318	—
<i>Coccodiella melastomatidis</i> (Lév.) I. Hino & Katum.	—	Sordariomycetes	Phyllachorales	U78543	—
<i>Coccoltrema cucurbitula</i> (Mont.) Müll. Arg.	Argentina, Prov. Rio Negro, 1999, <i>Vobis</i> (ESS 20862)	—	—	AF274114	AF274092
<i>Coccoltrema pocillarium</i> (Cumings) Brodo	USA, Alaska, <i>Printzen</i> (ESS 20863)	—	—	AF274113	AF274093
<i>Conotrema populorum</i> Gilenstam	—	Lecanoromycetes	Ostropales	U86582	—
<i>Diatrype disciformis</i> (Hoffm.) Fr.	—	Sordariomycetes	Xylariales	U32403	—
<i>Diploschistes rampoddensis</i> (Nyl.) Zahlbr.	Papua New Guinea, <i>Aptroot 39679</i> (hb. Lumbsch)	Lecanoromycetes	Ostropales	AF274111	AF274094
<i>Diploschistes thunbergianus</i> (Ach.) Lumbsch & Vezda	Australia, New South Wales, 1997, <i>Eldridge 3800</i> (hb. Lumbsch)	Lecanoromycetes	Ostropales	AF274112	AF274095
<i>Eremascus albus</i> Eidam	—	Eurotiomycetes	Eurotiales	M83258	—
<i>Eurotium rubrum</i> W. Bremer	—	Eurotiomycetes	Eurotiales	U00970	—
<i>Fonsecaea pedrosoi</i> (Brumpt) Negróni	—	Chaetothryomycetes	Chaetothryiales	L36997	—
<i>Gyalecta ulmi</i> (Sw.) Zahlbr.	—	Lecanoromycetes	Gyalectales	AF088237	—
<i>Halosphaeriopsis mediosetigera</i> (J.W. Cribb & Cribb) T.W. Johnson	—	Sordariomycetes	Halosphaeriales	U32420	—
<i>Helvella lacunosa</i> Afzel.	—	Pezizomycetes	Pezizales	U42654	—
<i>Hypomyces chrysospermus</i> Tul. & C. Tul.	—	Sordariomycetes	Hypocreales	M89993	—
<i>Lecanactis abietina</i> (Ach.) Körb.	—	Arthoniomycetes	Arthoniales	U23539	—
<i>Leotia lubrica</i> (Scop.) Pers.	—	Leotiomycetes	Leotiales	L37536	—
<i>Lepolichen coccophorus</i> (Mont.) Trevis	Argentina, Prov. Neuquén, 1999, <i>Messuti 2001</i> (hb. Lumbsch)	—	—	AF274110	AF274096
<i>Leucostoma persoonii</i> Höhn.	—	Sordariomycetes	Diaporthales	M83259	—
<i>Massaria platani</i> Ces.	—	Dothideomycetes	Pleosporales	AF164363	—
<i>Meliola juddiana</i> F. Stevens	—	Sordariomycetes	Meliolales	AF021793	—
<i>Ochrolechia parella</i> (L.) A. Massal.	France, Brittany, 1999, <i>Feige</i> (ESS 20864)	Lecanoromycetes	Pertusariales	AF274109	AF274097
<i>Ochrolechia szatalaensis</i> Verseghy	Argentina, Prov. Rio Negro, 2000, <i>Messuti 2008</i> (hb. Lumbsch)	Lecanoromycetes	Pertusariales	AF274108	AF274102
<i>Pertusaria amara</i> (Ach.) Nyl.	Germany, Rheinland-Pfalz, 2000, <i>Killmann</i> (ESS 20865)	Lecanoromycetes	Pertusariales	AF274104	AF274101

Table 1. (Continued).

Species	Collection	Class	Order	GenBank - SSU	GenBank - LSU
<i>Pertusaria erythrella</i> A. W. Archer	Australia, New South Wales, 2000, Archer (ESS 20866)	Lecanoromycetes	Pertusariales	AF274106	AF274100
<i>Pertusaria scaberula</i> A. W. Archer	Australia, New South Wales, 2000, Archer (ESS 20867)	Lecanoromycetes	Pertusariales	AF274105	AF274099
<i>Pilophorus acicularis</i> (Ach.) Th. Fr.	—	Lecanoromycetes	Lecanorales	AF085469	—
<i>Placopsis argillacea</i> (Knight) Malcolm & Vezda	New Zealand, Southland, 1997, Malcolm & Vezda (Vezda exs. 340) (hb. Lumbsch)	Lecanoromycetes	Agyriales	AF274107	—
<i>Placopsis gelida</i> (L.) Linds.	—	Lecanoromycetes	Agyriales	AF119502	—
<i>Pleospora herbarum</i> P. Karst.	—	Dothideomycetes	Pleosporales	U05201	—
<i>Pseudevernia cladonia</i> (Tuck.) Hale & Culb.	—	Lecanoromycetes	Lecanorales	AF088245	—
<i>Rhytidhysterion rufulum</i> (Spreng.) Speg.	—	Dothideomycetes	Patellariales	AF201452	—
<i>Rhytisma salicinum</i> (Pers.) Fr.	—	Leotiomycetes	Rhytismatales	U53370	—
<i>Sordaria fimicola</i> (Desm.) Ces. & de Not.	—	Sordariomycetes	Sordariales	X69851	—
<i>Sporormia lignicola</i> W. Phillips & Plowr.	—	Dothideomycetes	Pleosporales	U42478	—
<i>Squamarina lentigera</i> (Weber) Poelt	—	Lecanoromycetes	Lecanorales	AF088250	—
<i>Stictis radiata</i> (L.) Pers.	—	Lecanoromycetes	Ostropales	U20610	—
<i>Thelomma mammosum</i> (Hepp) A. Massal.	—	Lecanoromycetes	Lecanorales	U86697	—
<i>Trapelia involuta</i> (Taylor) Hertel	Germany, Nordrhein-Westfalen, 1999, Lumbsch (ESS 20868)	Lecanoromycetes	Agyriales	AF119499	AF274098
<i>Trapelia placodioides</i> Coppins & James	Germany, Nordrhein-Westfalen, 1999, Lumbsch (ESS 20869)	Lecanoromycetes	Agyriales	AF119500	AF274103
<i>Tuber melanosporum</i> Vittad.	—	Pezizomycetes	Pezizales	L37001	—
<i>Xanthoria elegans</i> (Link) Th. Fr.	—	Lecanoromycetes	Lecanorales	AF088254	—
<i>Xylaria hypoxylon</i> (L.) Grev.	—	Sordariomycetes	Xylariales	U20378	—

SSU rRNA gene: nu-SSU-0021-5' (Gargas & DePriest, 1996), nu-SSU-0402-5, nu-SSU-0819-5', nu-SSU-0852-3', nu-SSU-1750-3' (Gargas & Taylor, 1992), nu-SSU-1184-3' (Gargas et al., 1995), and nu-SSU-0553-3' (White et al., 1990); b) for the LSU rRNA gene: nu-LSU-0155-5' (=AL1R) (Döring et al., 2000), nu-LSU-0654-5' (=LR3R), nu-LSU-0635-3' (=LR3), nu-LSU-1125-3' (=LR6), nu-LSU-1432-3' (=LR7) (Vilgalys homepage). Cycle sequencing was executed with the following program: 25 cycles of 95°C for 30 s, 48°C for 15 s, 60°C for 4 min. Sequencing products were precipitated and dried before they were loaded on an ABI 377 (Perkin Elmer) automatic sequencer. Sequence fragments obtained were assembled with SeqMan 4.03 (DNASTar).

Sequence alignment

Analysis 1—To estimate the phylogenetic position of the Coccotremataceae with regard to the classes of ascomycetes we have aligned seven new SSU rDNA sequences with 32 sequences obtained from GenBank (Tab. 1). Sequences from GenBank were selected to ensure that at least two taxa of each class distinguished by Eriksson and Winka (1997) were included. Preliminary multiple alignments were generated using Clustal W (Thompson et al., 1994), and manually optimized. Missing data at the 5'- and 3'-end of partial SSU rDNA sequences were coded by '?'. Major insertions (one 217bp intron in the LSU of *Coccotrema pocillarium*, two 58bp and 355bp introns in the SSU of *Ochrolechia parella* and one 67bp intron in the SSU of *Pertusaria amara*) were excluded and will be analyzed elsewhere.

Analysis 2—To further evaluate the phylogenetic position of the Coccotremataceae, SSU and LSU rDNA sequences of nine Lecanoromycetes and three Coccotremataceae (Tab. 1) were aligned. Preliminary multiple alignments were generated using Clustal W (Thompson et al., 1994) and manually optimized. Missing data at the 5'- and 3'-end of partial SSU rDNA sequences were coded by '?'. Major insertions were excluded. Ambiguously alignable positions were eliminated.

Phylogenetic analysis

All alignments were analysed using the PAUP* 4.0 software package (Swofford, 1998). In analysis 1, a neighbor joining (NJ) analysis was performed for a rough estimation of the phylogenetic position of the Coccotremataceae within the ascomycetes. The NJ analysis employed the LogDet transformation (Lockhart et al., 1994), which is consistent for sequences with differing nucleotide frequencies. All invariant sites were excluded as necessary for LogDet transformation (Huson, 1998). The tree was rooted using two pezizalean taxa. Nonparametric bootstrap support (Felsenstein, 1985) for each clade was tested based on 10,000 replications, using the NJ bootstrap option of PAUP*4.0.

In analysis 2, parsimony analyses and comparisons of the trees obtained from the two molecular data sets were carried out with the Lecanoromycetous taxa only. The trees were rooted using two Ostropalean taxa. The Ostropales together with Agyriales are the sister-group to Pertusariales in the SSU rDNA tree (Fig. 1). Maximum parsimony (MP) trees were inferred using the branch-and-bound search option. Gaps were

treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap support (Felsenstein, 1985) for each clade was tested based on 2000 replications, using the branch-and-bound bootstrap option of PAUP*4.0. Phylogenetic trees were drawn using Treeview (Page, 1996). The consistency index, CI; (Kluge & Farris, 1969), retention index, RI; (Farris, 1989), and rescaled consistency index, RC; (Farris, 1989) were obtained from MacClade 3.07 (Maddison & Maddison, 1992). Data decisiveness, DD (Goloboff, 1991; Davis et al., 1998) was calculated for each of the data sets using M and S obtained from MacClade 3.07, and S' approximated by the average length of 1,000,000 randomly resolved trees as obtained from PAUP*.

Data and tree evaluation

In analysis 2, the two molecular data sets revealed differing results. We compared the two portions, the probability of the alternative topologies in each data set, and attempted to find reasons for the discrepancy in the results. Both portions of analysis 2 were examined using the following steps:

1. Phylogenetic signal: If a data set does not have a structure significantly different from random, no confidence can be placed in the resulting tree topology. Thus we examined the structure of the two data sets using a PTP test (Faith, 1991) as implemented in PAUP* using 10,000 random matrices to make sure that both data sets contain sufficient phylogenetic signal.

2. Long-branch attraction: To examine the possibility that the inferred phylogenetic relationships were due to long-branch attraction (Felsenstein, 1978), we employed a χ^2 -test for deviant nucleotide composition using Puzzle 4.0 (Strimmer & von Haeseler, 1996, 1997). The detection of long-branch attraction is problematic (Sanderson et al., 2000) and different strategies to investigate this phenomenon were proposed, including simulation (Sanderson et al., 2000) and regression and variance analyses (Lyons-Weiler & Hoelzer, 1997). We have chosen a simple approach to examine the possibility that long-branch attraction is the cause of discrepancy between the two portions in analysis 2. A χ^2 -test was also employed by Stiller & Hall (1999) to detect long-branch attraction.

3. Congruence testing: The congruence of both data sets was examined, employing the partition homogeneity test (Farris et al., 1994) as implemented in PAUP* 4.0. In this test the difference between the numbers of steps required by individual and combined analyses are calculated. This incongruence length difference (ILD) (Mickey & Farris, 1981) is estimated by comparing the ILD for the original partitions and a series of randomized partitions. Invariant characters were excluded before applying the test as recommended by Cunningham (1997).

4. Support for alternative topologies: The Kishino-Hasegawa (1989) test as implemented in PAUP* was employed to check the probability of trees alternative to the most parsimonious trees. The tests were generated by running parsimony analyses while enforcing topological constraints. We also compared the conclusiveness of the two tree topologies by comparing the ability of the data sets to support a single topology.

5. Splits decomposition: This method was used to visualize any different or conflicting phylogenetic signals in the data sets not visible in the most parsimonious trees. If conflicting signals

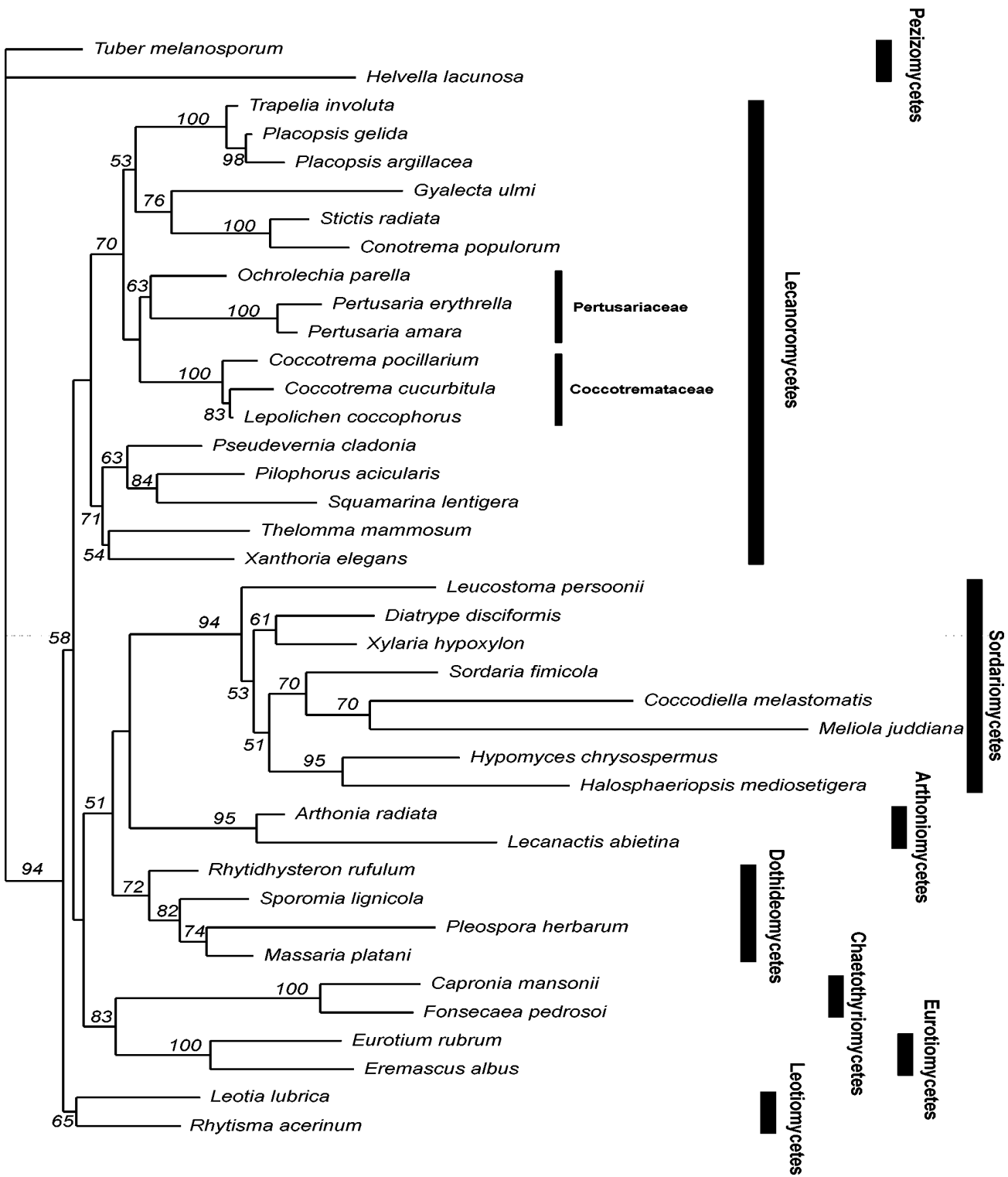


Fig. 1. LogDet NJ phylogram of the SSU rDNA of Pezizomycotina as obtained using PAUP*. Bootstrap values above 50% are included. The class placement of the genera is indicated at the margin.

are present in a data set, the splits decomposition graph exhibits a polygonal rather than tree-like topology. Polygonal topologies may be due to reticulate evolution (Dopazo et al., 1993), noise in the data or other reasons. Hamming distances were used as distance transformation. A full description of splits decomposition can be found elsewhere (Bandelt & Dress, 1992; Huson, 1998).

6. Analysis of the distribution of changes and amount of homoplasy in characters: MacClade version 3.07 (Maddison & Maddison, 1992) was used to plot the distribution of changes in the two data sets over the alignments. Further, the retention index (RI) was plotted for the molecular characters using MacClade to show the amount of homoplasy in the informative characters. All uninformative characters were excluded prior to plotting.

7. Calculation of the ideal nucleotide substitution rate for the phylogeny of Coccotremataceae and allied families: The results of the analysis of the distribution of changes over the alignment suggested that the SSU rDNA data are not variable enough to contain sufficient phylogenetic information. We em-

ployed an examination of phylogenetic trees using likelihood calculations based on Markov-process models of nucleotide substitution (Goldman, 1998) to further evaluate this suggestion. The ideal nucleotide substitution rate for the phylogeny of the group examined was calculated using the computer program Edible (Massingham & Goldman, in press). Because of computational effort the ostropalean taxa were excluded from this analyses. The maximum likelihood analyses employed the Jukes-Cantor model of DNA substitution (Jukes & Cantor, 1969).

Results

Initial SSU analysis to locate the phylogenetic position of the Coccotremataceae (Analysis 1)

We obtained sequences of SSU rDNA varying in length between 1239 (in *Pertusaria erythrella* and *P. scaberula*) and 1770 bp (in *Placopsis argillacea*). More than 95 %

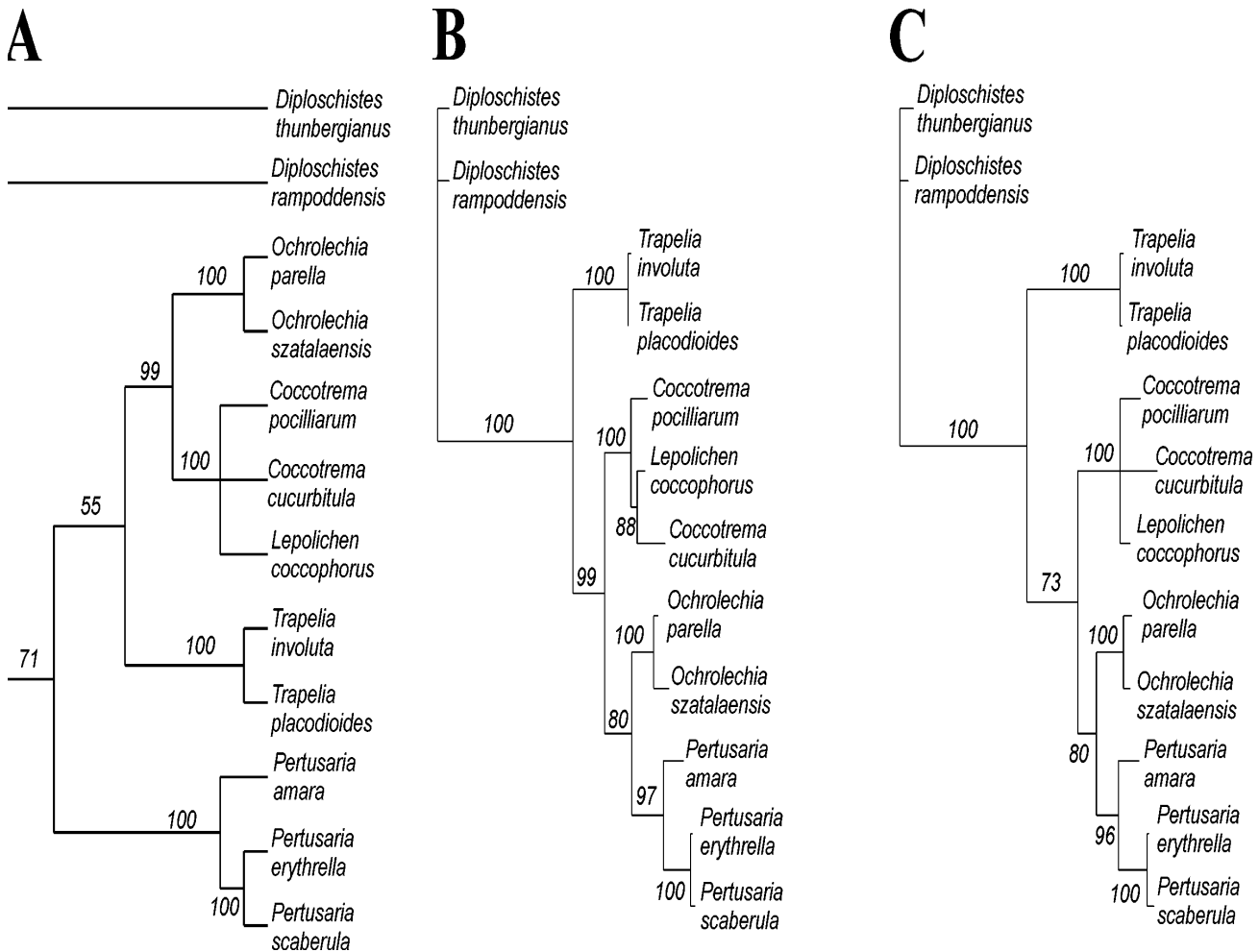


Fig. 2. Phylogenetic trees of the separate analyses of the SSU and LSU rDNA portions and of the combined data set. A. Strict consensus tree of the two most parsimonious trees obtained from the SSU rDNA portion. B. Most parsimonious tree obtained from the LSU rDNA portion. C. Most parsimonious tree obtained from the combined data set (SSU and LSU rDNA portions). All analyses were run in a branch and bound search using PAUP*. Bootstrap support values above 50% are shown.

of the sequence lengths were sequenced in both directions in all species. Most sequences retrieved from GenBank were of about a similar length, but some were much shorter; these include *Arthonia radiata* (762 bp) and *Lecanactis abietina* (835 bp).

Sequences of the 39 taxa were aligned to produce a matrix of 1807 nucleotide-position characters. The NJ tree obtained employing the LogDet transform is shown in Fig. 1. The Coccotremataceae cluster near the Pertusariaceae within the Lecanoromycetes with apothecia. However, there is no bootstrap support for a close relationship of the two families. A MP search (with 200 random sequence additions) revealed a topology (tree length 1537 steps) identical to the NJ analysis (results not shown), but no bootstrap values were calculated for the MP analysis.

Combined analyses of the phylogenetic relationships of the Coccotremataceae (Analysis 2)

We identified 1239 (in *Pertusaria erythrella* and *P. scaberula*) to 1754 bp (in *Lepolichen coccophorus*) of the SSU rRNA gene from the different species and obtained sequences of LSU rDNA in a length varying from 1205 (in *Coccotrema pocillarium*) to 1220 bp (in *Pertusaria erythrella* and *P. scaberula*). More than 95 % of the sequence lengths were sequenced in both directions in all species. The sequences were aligned to produce a matrix of 1401 nucleotide position characters in the SSU and 1225 for the LSU portion of the second data set. Regions that showed ambiguous alignment were excluded. However, these were few: 14 characters in the SSU and 27 positions in the LSU alignment were eliminated, resulting in 1387 characters (120 informative sites) for the SSU and 1198 characters (190 informative sites) for the LSU portion of this data set. The alignments are available in TreeBASE SN480 (<http://herbaria.harvard.edu/treebase/>).

Analysis of the SSU portion of analysis 2 resulted in two MP trees, 216 steps long, CI=0.81, RI=0.85, RC=0.73, and DD=0.75. The strict consensus tree of the two MP trees is shown in Fig. 2A. The Coccotremataceae appear as sister group to the genera *Ochrolechia* and *Trapelia*, but this relationship lacks bootstrap support (55 %). The Agyriales (as circumscribed by Lumbsch et al., in press) (*Trapelia*) cluster within the Pertusariales (as circumscribed by Ott and Lumbsch, 2000) and the Pertusariaceae, as currently circumscribed, are paraphyletic. The Pertusariales s. lat. (including Agyriales) has a moderate bootstrap support of 71%.

In the analysis of the LSU portion, the MP analysis revealed one MP tree (Fig. 2B), 332 steps long, with CI=0.79, RI=0.83, RC=0.68, and DD=0.80. In this tree, the Coccotremataceae are a sister group of the Pertusariaceae which are monophyletic. The Pertusariaceae have

a moderate statistical support of 80 %, and the Pertusariales including the Coccotremataceae have a strong support of 100 %. The Agyriales (*Trapelia*) are the sister group to the Pertusariales (incl. Coccotremataceae).

The combined analysis revealed one MP tree, 580 steps long, with CI=0.82, RI=0.86, RC=0.71, and DD=0.81 (Fig. 2C). The tree topology is almost identical with the analysis of the LSU portion, but the bootstrap values are slightly lower (Pertusariales including Coccotremataceae with 73% bootstrap support) and the relationship within the Coccotremataceae remains unresolved.

Comparison of the SSU and LSU portions of analysis 2

The tree topologies of the two portions examined gave different results regarding the monophyly of the Pertusariales and Pertusariaceae. Thus we further examined the two portions beyond a standard cladistic analysis to find out which phylogenetic hypothesis is more likely to reflect the true phylogeny. The standard indices (CI, RI, RC) and the data decisiveness (DD) do not show any significant differences between the two portions. However, the number of parsimony-informative sites is much higher in the LSU than in the SSU data set.

1. Phylogenetic signal: The PTP test indicated that each data set had significant phylogenetic structure ($p=0.0001$ for the combined data set and both portions), suggesting that random noise is not a factor in the differences observed between the two portions of the data set.

2. Long-branch attraction: All sequences of the SSU and LSU portions passed the 5% - χ^2 -test, suggesting that long-branch attraction is not evident in the analyzed data sets. Since the branches in the part of the trees in question did not show any obvious length, we considered long-branch attraction not being of major importance in this case and did not further evaluate this phenomenon.

3. Congruence testing and combined analysis: The ILD test revealed that the SSU and LSU portions are congruent ($p=0.30$) and that they can be analyzed in a combined analysis. In the combined analysis, one MP tree was obtained which had a topology identical with the MP tree obtained from the LSU data set alone (tree not shown). The bootstrap values were very similar in both analysis, but tended to be lower in the combined analysis.

4. Kishino-Hasegawa test: While the SSU portion suggested a sister group relationship of the Coccotremataceae with *Ochrolechia* and the Agyriales, the LSU portion placed the Coccotremataceae + Pertusariaceae as sister group to the Agyriales. We employed the Kishino-Hasegawa test to examine whether the alternative topologies were likely in each and the combined sets. A placement of the Coccotremataceae as sister

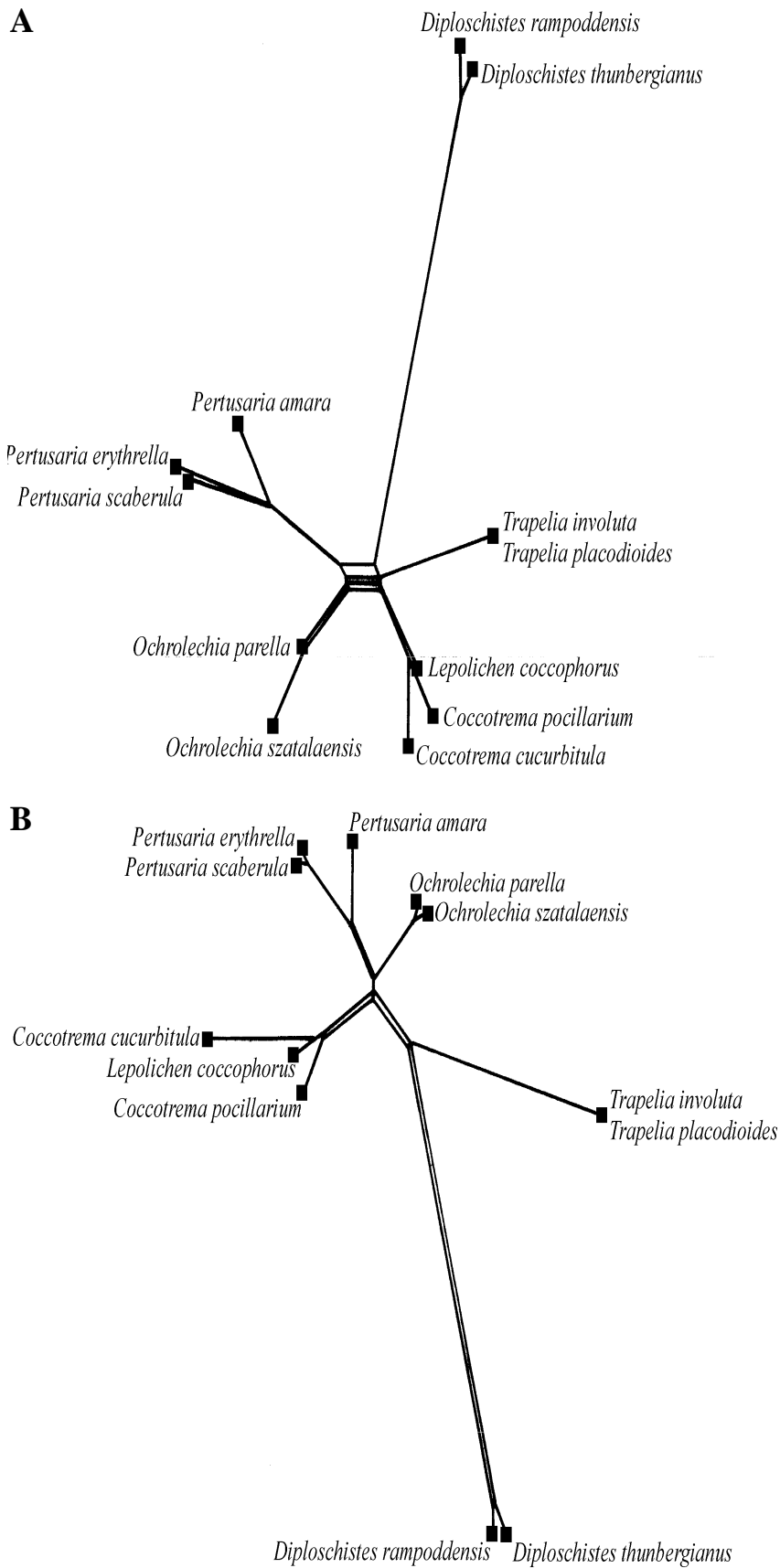


Fig. 3. Split decomposition graph of ten taxa (Agyriales and Pertusariales) of the combined data set. A. SSU rDNA portion. B. LSU rDNA portion.

group to *Ochrolechia* and *Agyriales* is rejected in the LSU portion (tree length 347 steps, $SD=5.815$, $p=0.0060^*$) and the combined data set (tree length 598 steps, $SD=6.921$, $p=0.0093^*$). A tree topology as suggested by the LSU portion cannot be rejected in the SSU analysis, and appears only slightly less likely (tree length 218 steps, $SD=3.317$, $p=0.7390$). The SSU portion is less powerful in discriminating between the two alternative topologies than the LSU portion and combined data set.

Regarding the distinction of the two genera within the *Coccotremataceae*, neither character set seems to contain sufficient phylogenetic signal to resolve the phylogeny. In the SSU-MP tree the relationships appear as an unresolved polytomy, while the LSU portion suggest that *Lepolichen coccophorus* and *Coccotrema curbitula* are sister groups and hence a distinction of *Lepolichen* at generic rank would make *Coccotrema* pa-

raphyletic. However, the alternative topology placing *Lepolichen* basal to the two *Coccotrema* spp. cannot be rejected using the Kishino-Hasegawa test (data not shown).

5. Splits decomposition: The Kishino-Hasegawa test showed that the two alternative tree topologies have similar likelihood in the SSU portion and thus we suppose that conflicting phylogenetic signals were present in the SSU data. To further evaluate this, we employed splits decomposition. The results of these analyses for the SSU and LSU portions of analysis 2 are shown in Fig. 3. The split decomposition graph of the SSU data clearly shows a polygonal topology regarding the relationship of *Ochrolechia*, *Pertusaria*, the *Coccotremataceae*, *Agyriales*, and *Ostropales*, showing that the relationships between these groups cannot be evaluated with this data set (Fig. 3A). Such box-like topologies are not evident in the analysis of the LSU portion (Fig. 3B).

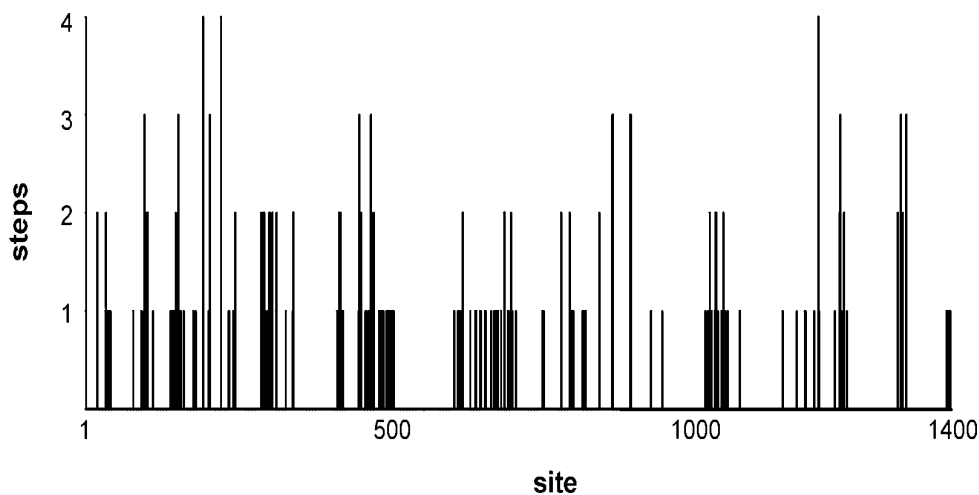


Fig. 4. Distribution of changes in SSU rDNA sequences in the Lecanoromycetes data set. This plot was generated using the CHART option of MacClade version 3.03 (Maddison & Maddison, 1992).

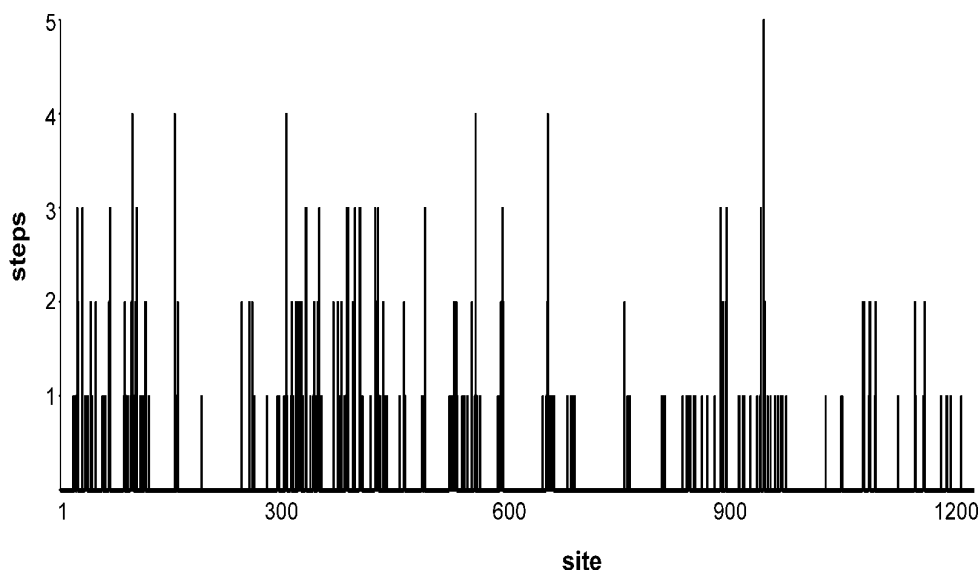


Fig. 5. Distribution of changes in LSU rDNA sequences in the Lecanoromycetes data set. This plot was generated as described in the legend of Fig. 4.

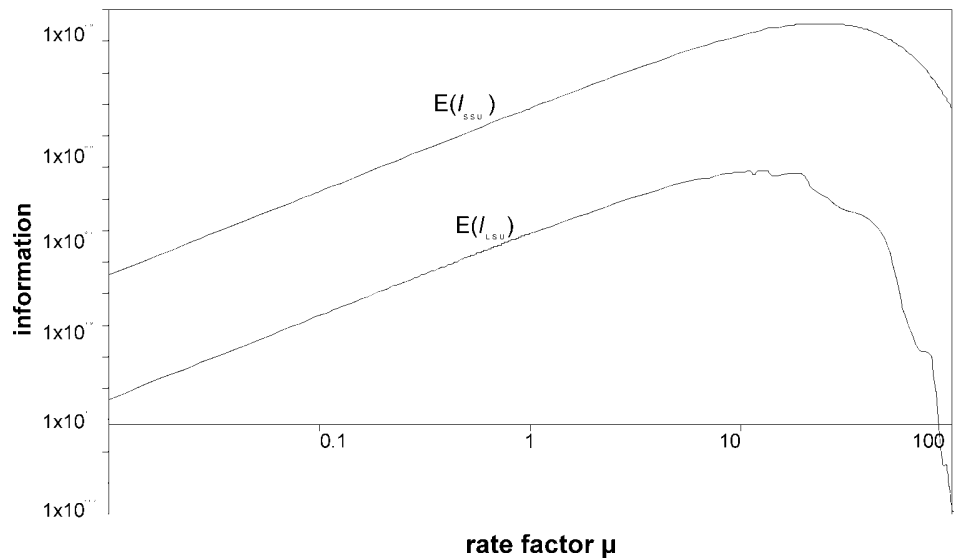


Fig. 6. Expected information $|E(I)|$ of the SSU and the LSU rDNA trees plotted against rate factor μ (both scaled logarithmically).

6. Analysis of the distribution of changes and amount of homoplasy in characters: The steps in sites over the two alignments is shown in Figs. 4-5. The SSU (Fig. 4) portion shows considerably less variation than the LSU (Fig. 5) portion, both in number of steps and in amount of variable sites. The comparison of the RI values of parsimony-informative characters shows that the amount of homoplasy is relatively higher in the informative sites of the SSU portion than in the LSU portion (c. 30% of the characters in the SSU and 26% in the LSU data set show homoplasy). This is evident when characters with $RI=0$ are compared; these are c. 7% in the SSU and c. 2% in the LSU data set. We examined whether homoplasy is responsible for the potentially misleading phylogenetic signal in the SSU portion and re-ran the MP analysis including only characters with $RI=1$ (trees not shown). The 50%-bootstrap tree of the SSU portion does not support any relationship above the genus (with the exception of *Coccotrema* and *Lepolichen*), while the bootstrap tree of the LSU portion is identical in topology with the LSU-MP tree obtained from the complete data set. Also the bootstrap values are similar or higher than in the entire data set analysis.

7. Calculation of the ideal nucleotide substitution rate for the phylogeny of Coccotremataceae and allied families: The analysis of the two data sets suggested that the SSU portion may not be variable enough to contain sufficient phylogenetic information for the study of the phylogeny of the Coccotremataceae and related families. Using a likelihood based approach we calculated the ideal nucleotide substitution rate for the SSU and LSU trees. The results shown in Fig. 6 reveal that neither gene has an ideal variability. For the SSU portion, the amount of information per site relating to all branch lengths, $|E(I_{SSU})|$, has its maximum at 24; this

means that the ideal gene for this phylogenetic question would be one with a nucleotide substitution rate 24 times higher than that of the SSU gene. For the LSU portion, the ideal gene would be one with a nucleotide substitution rate 13 times higher than that of the LSU gene, since $|E(I_{LSU})|$ has its maximum at 13.

Discussion

Utility of the SSU and LSU data sets

There are different approaches regarding the analyses of multiple data sets. While some authors prefer separate analyses (e.g., Pesole et al., 1991, Shaffer et al., 1991), others argue for the simultaneous analysis of the combined data, the total evidence approach (e.g., Miyamoto, 1985; Kluge, 1989). However, combining incongruent data sets may yield erroneous estimates of relationships (Bull et al., 1993). Therefore, different tests for the congruence of data sets have been developed (e.g., Bull et al., 1993; Rodrigo et al., 1993; Farris et al., 1994) to decide whether data are congruent. We have used the ILD test (Mickey & Farris, 1981; Farris et al., 1994; Cunningham, 1997), the results suggest that both data sets can be analyzed in a combined approach. The tree topology derived from the combined data set (Fig. 3C) does not differ from that of the LSU data set only. According to this observation it would be advisable to combine data sets because one data set alone (in this case the SSU portion) produces an incorrect phylogenetic estimate. However, one should keep in mind that the SSU data set does not perform very well in the comparative tests. It is too invariable to contain sufficient phylogenetic information as shown in the likelihood based calculation of the ideal nucleotide substitution rate, the low amount of variable

characters indicated by the plot in Fig. 4, and the lack of any bootstrap support above the genus level in a tree obtained from an analysis of characters with $RI=1$. The lack of support for relationships in the Lecanoromycetes due to insufficient variability in the SSU rDNA has already been reported (e.g., Stenroos & DePriest, 1998; Wedin & Döring, 1999). The potentially misleading phylogenetic signal for a placement of the Agyriales within the Pertusariales is due to homoplasy in the parsimony-informative characters that is higher than in the LSU portion of the data set in analysis 2. This signal overshadows the phylogenetic signal supporting a monophyletic Pertusariales including the Coccotremataceae. This signal is also present in this data set as indicated by the split decomposition graph (Fig. 3A) and the Kishino-Hasegawa test with lack of power to reject the alternative tree topology. The indices (CI, RI, RC, DD) developed to calculate the amount of homoplasy do not provide any help either, since the overall amount of variation in the data set is very small. However, this small amount of variation seems to be responsible for the homoplasious characters in the SSU portion yielding an incorrect phylogenetic estimate. The restrictions of the SSU rDNA for phylogenetic reconstructions are well known, since this gene consists of highly conserved and more variable regions resulting in different historical signals. Homoplasy in the variable regions makes resolution of relationships difficult (Soltis et al., 1999).

Taking these drawbacks of the SSU data set into account, we suppose that the phylogenetic signal in the SSU data set has negative effects on the combined tree. Since the LSU data set performs better in the comparative tests and its resulting tree topology is the same as in the combined tree, we think that the combined tree only has the correct topology because the correct phylogenetic signal in LSU data set overshadows the incorrect phylogenetic signal in SSU data set. In this case we see no advantage in combining the two data sets. The only result would be a weakening of the bootstrap values caused by erroneous phylogenetic signals. We suggest that when multiple data sets support different tree topologies, the reasons for this discrepancy should be evaluated beyond an examination of the congruence of the data sets. Data sets should only be combined if each character set has variability, large enough to contain sufficient phylogenetic information and small enough not to contain too much homoplasy. Different methods to estimate evolutionary rates for molecular data sets are available for systematists (e.g., Goldman, 1998; Yang, 1998). Also if only one data set is available, the analyses for tree evaluation performed in this study may be helpful to test the reliability of the results of the phylogenetic analysis.

The LSU data set also does not have an ideal nucleotide substitution rate, but its variability is sufficient to allow discrimination of monophyletic groups with

good bootstrap support that are robust to likelihood based comparisons with alternative topologies. The splits decomposition graph indicated that no conflicting signals are present in the LSU portion and the analysis of characters with $RI=1$ which revealed an identical topology as the analysis of the entire data set, suggested that homoplasy is not a serious problem in the LSU portion. Judging from these results, we are confident that the MP tree obtained from the LSU portion alone represents a reasonable phylogenetic hypothesis to base our classification of these Lecanoromycetes.

The phylogenetic position of the Coccotremataceae and the nature of its ascomata

The example of the Coccotremataceae shows that even a comprehensive study of the morphological characters (such as ascus structure, ascoma ontogeny, etc.) does not always allow a satisfying classification. The molecular data contain additional information which may help to illuminate the phylogeny of this group of ascomycetes.

Although the SSU data set proved to be of limited use for the evaluation of the phylogenetic relationships within the Lecanoromycetes, it provided evidence that the Coccotremataceae belong to this class. Thus an interpretation of their ascomata as modified apothecia, as already suggested by Henssen (1976), seems appropriate, given that the ascomata of the Coccotremataceae are not the result of convergent evolution.

However, the use of molecular markers may also yield contradicting phylogenetic hypotheses as our analysis of LSU and SSU data shows. Whereas the SSU portion does not resolve the placement of the Coccotremataceae in either Pertusariales or Agyriales, the LSU portion and the combined tree support a placement of the Coccotremataceae within the Pertusariales. Since the LSU tree has higher bootstrap values than the SSU tree and the data set performs better in the comparative tests it might be considered to move the family from its uncertain status to this order. However, such a classification remains tentative, until more taxa and larger data sets have been analyzed. Morphological and chemical characters which would support such a classification include large, hyaline, and thick-walled ascospores and the presence of β -orcinol depsidones.

Neither of the three data sets (SSU, LSU, SSU+LSU) has sufficient variability to allow a statement on the generic concept within the Coccotremataceae. In the SSU and the combined tree the relationship is unresolved and although in the LSU MP tree *Coccotrema* is paraphyletic, a monophyletic *Coccotrema* cannot be rejected. The two genera of the Coccotremataceae are very similar in morphology and chemistry, and mainly differ in their thallus organization. *Coccotrema* contains

a crustose thallus, while the monotypic *Lepolichen* has terete lobes and fibrillar rhizines (Galloway & Watson-Gandy, 1992). Further studies on more variable genes (ITS, mt SSU rDNA) are currently undertaken to evaluate the generic concept in the *Coccotremataceae*.

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