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Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships based on nuclear ribosomal RNA small and large subunits

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Abstract

Despite various morphological and anatomical similarities, the two orders Gyalectales (lichenized ascomycetes) and Ostropales (lichenized and non-lichenized ascomycetes) have been considered to be distantly related to each other and their position within the Ascomycota was unsettled. To estimate relationships within these groups and their respective phylogenenetic placement within the Ascomycota, we analyzed DNA sequences from the nuclear small and large subunit ribosomal RNA genes using Maximum Parsimony, Maximum Likelihood, and Bayesian statistics with Markov chain Monte Carlo algorithms. Support for internal branches estimated with bootstrap was compared to Bayesian posterior probabilities. We report here that the Ostropales, in their current circumscription, are paraphyletic, and that the Ostropales s.l. include the Gyalectales and Trapeliaceae. The Unitunicate Ascohymenials are redelineated to include the Ostropales s.l., as defined here, and the Baeomycetaceae. *Dimerella* and *Coenogonium* are congeneric, and *Petractis thelotremella* and *P. hypoleuca* are reunited with members of the genus *Gyalecta*. In addition to requiring less computational time, Bayesian inference of phylogeny recovered the same topology as a conventional heuristic search using Maximum Likelihood as the optimization criterion and seems superior to bootstrapping in estimating support for short internal branches.

Keywords: Ostropales; Gyalectales; Fungi; Ascomycetes; Lichens; Phylogeny; Maximum Likelihood; Maximum Parsimony; Bayesian inference; Markov chain Monte Carlo; Small subunit (SSU); Large subunit (LSU); Nuclear ribosomal DNA

1. Introduction

The Ascomycota, characterized by a saclike ascus containing haploid spores, is the largest of the four phyla within the fungi kingdom. Approximately 98% of all lichen-forming fungi are ascomycetes (Honegger, 1996), also named "ascolichens." These lichenized ascomycetes account for about 42% of all known ascomycete species (Hawksworth et al., 1995), which means that about one-fifth of all extant known species of fungi are lichenized. A substantial move towards an integral classification of both lichenized and non-lichenized ascomycetes started to take shape with Luttrell's work in 1951. In 1971, lichen-forming fungi were included for the first time in both the "Index of Fungi" and "Dictionary of the Fungi," and it was only in 1981 that lichens were no longer recognized as a "group" distinct from fungi in the International Code of Botanical Nomenclature (Hawksworth and Hill, 1984).

Concurrent to this relatively recent and essential integration of lichenized and non-lichenized ascomycetes within one system of classification, there was an increasing reluctance to use a supraordinal classification (classes) within the phylum Ascomycota (Eriksson, 1981; Hafellner, 1988; Hawksworth, 1985). The classification of the Ascomycota in the seventh edition of the "Dictionary of the Fungi" (Hawksworth et al., 1983) consisted of no more than a list of 39 orders, without any class-level organization. Morphological and anatomical characters alone seem to be insufficient to

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establish a stable supraordinal classification of the Ascomycota. Moreover, the homology of phenotypic features was often doubtful and misleading because of the lack of a rigorous phylogenetic context. Consequently, several conflicting classifications have been proposed (Barr, 1983; Eriksson, 1981, 1982; Eriksson and Hawksworth, 1993; Nannfeldt, 1932).

Despite the advent of molecular phylogenetics after the publication of the seventh edition of the "Dictionary of the Fungi," the situation did not improve significantly. The classification of the Ascomycota reported in the eighth edition of the "Dictionary of the Fungi" (Hawksworth et al., 1995) consisted of 46 orders still without any higher taxonomic categories, but with the recommendation that for general purposes it remained pragmatic to use only ordinal names. Moreover, the delimitation of many of these orders remained highly uncertain. The first molecular phylogenies of the Ascomycota were based exclusively on the nuclear small subunit ribosomal DNA (SSU rDNA), but the phylogenetic signal was limited to generating trees with very low level of confidence for deep relationships within the Euascomycetes (Berbee et al., 2000; Berbee and Taylor, 1995; Spatafora, 1995). The level of confidence for these deep internodes did not really improve when the nuclear large subunit ribosomal DNA (LSU rDNA) was analyzed separately from the SSU rDNA (Platt and Spatafora, 2000; Suh and Blackwell, 1999).

This unfortunate situation in the phylum Ascomycota has been impeding taxonomic work at all levels. The Gyalectales–Ostropales complex, including lichenized and non-lichenized genera with functionally unitunicate asci (Luttrell, 1951) and ascohymenial ascoma development (Nannfeldt, 1932), is no exception. Both the Gyalectales and the Ostropales were listed within the "Unitunicate Ascohymenials" in Tehler's (1996) classification of lichenized fungi. Despite the striking similarities between the two orders (ascospores, ascoma ontogeny, and anatomy), they have been considered being distantly related (Henssen and Jahns, 1974). However, recent studies based on combined phylogenetic analyses of the nuclear SSU and LSU rDNA (Bhattacharya et al., 2000; Lutzoni et al., 2001) support a very close relationship between members of the Ostropales and Gyalectales. An exhaustive combined phylogenetic study of the SSU and LSU rDNA at the ordinal level, including representative species from most of the lichenized and non-lichenized major lineages of Ascomycota, is needed to provide a skeletal phylogenetic structure upon which mycologists could build a stable and evolutionary meaningful classification. Such core SSU and LSU alignments amenable to combined phylogenetic analyses are now available for 52 species of the Ascomycota (Lutzoni et al., 2001) selected from 24 of 46 orders listed by Hawksworth et al. (1995) and representing about 75% of the species diversity within the Ascomycota. The combined analysis of these two data sets coupled with a Bayesian Markov chain Monte Carlo approach is sufficient to detect the monophyly of most major lineages of ascomycetes with a high level of confidence, including the Unitunicate Ascohymenials. To these 52 species, we added eight species classified within the Gyalectales or the Ostropales to confirm the phylogenetic placement of these two orders within the Ascomycota and their respective circumscription.

The order Gyalectales was introduced by Henssen and Jahns (1974), but not formally established until 1986 (Hawksworth and Eriksson, 1986). According to Eriksson and Hawksworth (1998), this order consists of one family (Gyalectaceae) and nine genera (*Belonia*, *Bryophagus*, *Coenogonium*, *Cryptolechia*, *Dimerella*, *Gyalecta*, *Pachyphiale*, *Ramonia*, and *Semigyalecta*). The first two genera were listed as doubtful members of the family. Based on morphological and anatomical features, *Dimerella* has recently been united with *Coenogonium* (Lücking and Kalb, 2000). The genus *Petractis* was originally part of the Gyalectales (Henssen and Jahns, 1974; Vězda, 1965), but is listed as a doubtful genus within the Stictidaceae–Ostropales by Eriksson and Hawksworth (1998).

The order Ostropales, erected by Nannfeldt (1932), originally included a single family, the Ostropaceae, and was based mainly on features of the ascus and ascospores. All of its members were non-lichenized until Gilenstam (1969) deduced that the lichenized genus *Conotrema* Tuck., formerly placed in the Diploschistaceae, is closely related to *Stictis* Pers., a genus classified within the Ostropales. He considered the possibility that they were congeneric, but decided to classify them as separate genera within the Stictidaceae. The obvious similarities in many characters (ontogeny of ascoma, development of ascospores and conidia, as well as ascus anatomy) between the Ostropales and the Diploschistaceae were formally taken into account by Henssen and Jahns (1974). In addition to the Ostropaceae, Stictidaceae, and Asterothyriaceae, Henssen and Jahns (1974) also included the Thelotremataceae (suborder Ostropinae, incl. *Thelotrema*, *Diploschistes*, *Ocellularia*, *Chroodiscus*, *Lepotrema*, and *Phaeotrema*) and Graphidaceae (suborder Graphidinae, incl. *Graphis*, *Graphina*, *Phaeographis*, *Sarcographa*, *Gyrostomum*, *Glyphis,* and *Acanthotheciopsis*) into the Ostropales. Since then, the circumscription of this order has been continuously under discussion (Eriksson and Hawksworth, 1993; Hawksworth et al., 1995; Sherwood, 1977). The recent "Outline of the Ascomycetes" (Eriksson and Hawksworth, 1998) lists eight families within the Ostropales: Asterothyriaceae, Gomphiliaceae, Graphidaceae, Odontotremataceae, Phaneromycetaceae, Solorinellaceae, Stictidaceae (including *Petractis*), and Thelotremataceae. A phylogenetic analysis based on SSU rDNA sequences (Winka et al., 1998) from representative

species of *Stictis*, *Conotrema*, *Cyanodermella* (Stictidaceae), *Diploschistes* (Thelotrematacеae), and *Graphis* (Graphidaceae) confirms the monophyly of the Ostropales s. str. However, the taxon sampling of this study was insufficient for further taxonomical conclusions.

The main goal of our study was to reconstruct the phylogenetic relationships of members of the Gyalectales and Ostropales within a broad selection of lichenized and non-lichenized ascomycetes. The specific objectives were to provide a better estimation of their phylogenetic position within the Ascomycota, to reveal relationships between a number of taxa currently included in these two orders, and to test the monophyletic status of both orders.

Phylogenetic analyses of the SSU and LSU rDNA were conducted using Maximum Parsimony (MP) and Maximum Likelihood (ML) as optimality criteria. Bootstrap support (BS; Felsenstein, 1985) and Bayesian/Markov chain Monte Carlo tree sampling (B/MCMC; Larget and Simon, 1999; Li, 1996; Mau, 1996; Mau and Newton, 1997; Mau et al., 1999; Rannala and Yang, 1994) were used to estimate levels of confidence associated with relationships revealed by these two phylogenetic searches. We compared the results from the BS and B/MCMC methods to gain some insight into their respective properties.

2. Materials and methods

2.1. Taxon sampling

To determine the phylogenetic placement and circumscription of the Gyalectales and Ostropales, two combinable core SSU and LSU alignments for the phylogenetic study of the ascomycetes generated by Lutzoni et al. (2001) were used to provide a basic selection of 52 species representing all main ascomycete orders known to include lichenized species (13 out of 15 orders) and nearly all main orders of Ascomycota known to include only non-lichenized species (11 out of 31 orders). These alignments also include two basidiomycetes as outgroups for a total of 54 species. To this core phylogenetic framework (Lutzoni et al., 2001), we added eight species belonging to the Gyalectales or the Ostropales for which we sequenced the DNA of both molecules, for a total of 62 species (Table 1).

To further explore relationships within these two orders, a second set of analyses was restricted to the LSU rDNA of 15 species classified within the Unitunicate Ascohymenials, using two *Acarospora* species as outgroups. To these 17 species selected from the initial 62 species, 11 LSU sequences were added, representing one additional *Petractis* species, four *Gyalecta* species, three *Dimerella* species, and three additional genera (*Chroodiscus*, *Ocellularia*, and *Phaeographina*), for a total of 28 LSU sequences (Table 1). All new SSU and LSU sequences were generated by F.K., with the exception of the LSU sequence for *Gyalecta leucaspis*, which was provided by Imke Schmitt, University of Essen, Germany. Although recently synonymized with *Coenogonium* (Lücking and Kalb, 2000), the genus name *Dimerella* was used here to reveal for the first time relationships among species belonging to what was recognized as two separate genera for 120 years, previous to the work of Lücking and Kalb.

2.2. DNA isolation, amplification, and sequencing

Lichen tissue was ground after deep-freezing in liquid nitrogen with a Mini-Beadbeater-8 (BioSpec Products, Bartlesville, OK, USA) together with a small amount of zirconium beads (0.7 mm). DNA was isolated using the Puregene Kit (GENTRA Systems) following manufacturer's protocol for filamentous fungi. Quality and quantity of DNA isolates were examined on a TBE 1% agarose gel. Symmetric PCR was performed with different DNA concentrations targeting 1.0 and 1.4 kb fragments at the 5′-end of the SSU and LSU rDNA genes, respectively. Symmetric PCR amplifications were done using the following primers: (1) SSU: NS17, NS21, NS22, NS24, nssu131, nssu97a, and nssu97b (Table 2); (2) LSU: LR7, LR0R, LIC15R, LIC2044, and LIC24R (Table 2).

PCR conditions were varied depending on quality and quantity of the isolated DNA and the quality of prior amplifications. Details of the different reaction conditions are available by request to F.K. The PCR products were purified by cutting the bands out of a 1% low melt agarose TAE gel and incubating with 1 μl Gelase (0.2 U/μl) at 42 °C for 1–2 h. Sequencing reactions were performed in a final 10 μl volume using BigDye Terminator (ABI PRISM, Perkin–Elmer) and following manufacturer's instructions. In addition to the PCR primers we used for symmetric amplifications, the following primers were used for cycle sequencing reactions: (1) SSU: SR7, SR7R, SR11R, nssu634, nssu897R, nssu1088, nssu1088R, NS4, and NS23 (Table 2); (2) LSU: LR3, LR5, LR6, LR3R, and LR5R (Table 2).

Sequenced products were precipitated with 10 μl deionized sterile water, 2 μl of 3 M NaOAc, and 50 μl of 95% EtOH. Polyacrylamide gel electrophoresis was conducted using Long Ranger Single Gel Packs (FMC Bioproducts) and an ABI 377A automated DNA sequencer (Perkin–Elmer, Applied Biosystems). Sequence fragments were subjected to BLAST searches to verify their identity and assembled using Sequencher 3.0.

2.3. Phylogenetic analyses

Phylogenetic analyses were carried out on several personal computers (one PIII/450 MHz, four Athlon/

Table 1
Vouchers, culture, DNA source information, and GenBank accession numbers for nuclear small and large subunit ribosomal DNA sequences (SSU and LSU rDNA) used in this study. Provisional classification follows mostly Lutzoni et al. (2001)

Classification	Taxon	Source for SSU	SSU GenBank accession numbers	Source for LSU	LSU GenBank accession numbers
Ascomycota					
Euascomycetes					
Acarosporaceae	*Acarosporal complanata*	Reeb VR 10-VIII-97 st 4.1/2 (DUKE)[a]	AF356653	Reeb VR 10-VIII-97 st 4.1/2 (DUKE)	AF356654
	Acarospora cf. dissipata	Reeb VR 12-X-97/11 st 4.1 (DUKE)	AF356655	Reeb VR 12-X-97/11 st 4.1 (DUKE)	AF356656
Unitunicate Ascohymemals					
Baeomycetaceae	*Baeomyces placophyllus*	Lutzoni 97.06.29-4 (DUKE)	AF356657	Lutzoni 97.06.29-4 (DUKE)	AF356658
Trapeliaceae	*Placopsis perrugosa*	Streimann 17.12.1993 (DUKE)	AF356659	Streimann 17.12.1993 (DUKE)	AF356660
	Trapeliopsis granulosa	Lumbsch & Feige 10.7.1994 (DUKE)	AF279414	Lumbsch & Feige 30.6.1992 (DUKE)	AF279415
Ostropales s.l.	*Petractis clausa*	Hafellner A 1/2 IAL3 96 (DUKE)	AF356661	Hafellner A 1/2 IAL3 96 (DUKE)	AF356662
	Petractis luetkemuelleri	**Nimis & Tretiach 2000, TSB 31659**	**AF465461**	**Nimis & Tretiach 2000, TSB 31659**	**AF465454**
	Bryophagus gloeocapsa	**Vězda 1998, TSB 30770**	**AF465456**	**Vězda 1998, TSB 30770**	**AF465440**
	Ocellularia alborosella	—	—	**Lücking 2000, 00-44**[b]	**AF465452**
Gyalectaceae	*Gyalecta jenensis A*	Lutzoni 98.08.17-6 (DUKE)	AF279390	Lutzoni 98.08.17-6 (DUKE)	AF279391
	Gyalecta jenesis B	—	—	**Nimis & Tretiach 1996, TSB 23635**	**AF465450**
	Gyalecta herrei	—	—	**Nimis & Tretiach 1993, TSB 18438**	**AF465449**
	Gyalecta truncigena	—		**Tretiach 1996, TSB 24274**	**AF465451**
	Gyalecta ulmi	**Scheidegger 30.05.1998 (DUKE)**	**AF465464**	**Scheidegger 30.05.1998 (DUKE)**	**AF465463**
	Gyalecta leucaspis	—	—	**Schmitt 08.06.2000**[c]	**AF465462**
	"***Petractis***" ***hypoleuca***	**Geletti & Tretiach 1995, TSB 20801**	**AF465460**	**Geletti & Tretiach 1995, TSB 20801**	**AF465453**
	"***Petractis***" ***thelotremella***	—	—	**Nimis & Tretiach 1996, TSB 22375**	**AF465455**
Coenogoniaceae	"***Dimerella***" ***subzonata A***	—	—	**Kauff 1998, pa03021998-506**	**AF465445**
	"***Dimerella***" ***subzonata B***	—	—	**Kauff 1998, pa00021998- 500**	**AF465446**
	"***Dimerella***" ***flavicans***	—	—	**Lücking 2000, DNA-79**[b]	**AF465444**
	Dimerella lutea	Ryan 31430 (ASU)	AF279386	Ryan 31430 (ASU)	AF279387
	Coenogonium disjunctum	**Kauff 1998, pa03021998-523**	**AF465458**	**Kauff 1998, pa03021998-523**	**AF465443**
	Coenogonium leprieurii	**Kauff 1998, pa04021998-522**	**AF465457**	**Kauff 1998, pa04021998-522**	**AF465442**
Graphidaceae–Thelotremataceae	*Diploschistes scruposus*	Reeb 12-X-97/10 st 4.1 (DUKE)	AF279388	Reeb 12-X-97/10 st 4.1 (DUKE)	AF279389
	Chroodiscus coccineus	—	—	**Lücking 2000, DNA-80**[b]	**AF465441**
	Graphina poitaei	**Lücking 2000, 00-34**[b]	**AF465459**	**Lücking 2000, 00-34**[b]	**AF465447**
	Phaeographina chrysocarpa	—	—	**Lücking 2000, 00-52**[b]	**AF465448**
Stictidaceae	*Stictis radiata*	—	U20610	JP222 (DNA[d], OSC, DUKE)	AF356663

Table 1 (*continued*)

Classification	Taxon	Source for SSU	SSU GenBank accession numbers	Source for LSU	LSU GenBank accession numbers
Eurotiomycetidae					
Umbilicariaceae	*Lasallia pensylvanica*	C.F. Culberson 22287 (DUKE)	AF356664	C.F. Culberson 22287 (DUKE)	AF356665
Chaetothyriales	*Capronia pilosella*	—	U42473	SMH 2565 (culture)[e]	AF279378
	Fonsecaea pedrosoi	—	L36997	ATCC 18658[f]	AF356666
	Exophiala jeanselmei	—	X80705	—	AF050271
Eurotiales	*Chromocleista malachitea*	—	D88323	—	AB000621
	Hamigera avellanea	—	D14406	—	AB000620
Pyrenulales	*Pyrenula cruenta*	Lutzoni 98.06.17-4 (DUKE)	AF279406	Lutzoni 98.06.17-4 (DUKE)	AF279407
Verrucariales	*Dermatocarpon americanum*	Merrill 0014313 (DUKE)	AF279383	Merrill 0014313 (DUKE)	AF279384
	Verrucaria pachyderma	Keller 1861, 16 Oct. 1994 (DUKE)	AF356667	Keller 1861, 16 Oct. 1994 (DUKE)	AF356668
Bitunicate ascohymenials					
Icmadophilaceae	*Dibaeis baeomyces*	—	AF113712	Lutzoni, 93.08.20-1 1/1 (DUKE)	AF279385
	Thamnolia subuliformis	—	AF113714	Brodo 28669B (DUKE)	AF356679
Caliciales	*Calicium viride*	Søchting 7475, 10-VII-1997 (DUKE)[g]	AF356669	Søchting 7475, 10-VII-1997 (DUKE)[g]	AF356670
Lecanorales	*Leptogium cyanescens*	FL1 (DUKE)	AF356671	FL1 (DUKE)	AF356672
	Placynthium nigrum	Lutzoni 98.08.17-11 (DUKE)	AF356673	Lutzoni 98.08.17-11 (DUKE)	AF356674
	Porpidia albocaerulescens	Spatafora 23-I-1994 (DUKE)	AF356675	Spatafora 23-I-1994 (DUKE)	AF356676
	Rhizocarpon disporum	Lutzoni 96.10.26-9 st. 1 (1/2) (DUKE)	AF356677	Lutzoni 96.10.26-9 st. 1 (1/2) (DUKE)	AF356678
	Stereocaulon paschale	Rceb STEPAS (DUKE)	AF279412	Reeb STEPAS (DUKE)	AF279413
	Sphaerophorus globosus	—	L37532	Lutzoni 93.07.21-3 (DUKE)	AF356680
Peltigerales	*Lobaria quercizans*	17-III-1992 (DUKE)	AF279396	17-III-1992 (DUKE)	AF279397
	Peltigera canina	Czyzewska 009205 (LOD-L)	AF356681	Miadlikowska & Lutzoni 2000	AF286822
Pertusariales	*Ochrolechia frigida* s.l.	Lutzoni 93.07.22-2 (DUKE)	AF279402	Lutzoni 93.07.22-2 (DUKE)	AF279403
	Pertusaria amara	Lutzoni 97.06.28 (DUKE)	AF356682	Lutzoni 97.06.28 (DUKE)	AF356683
Teloschistales	*Caloplaca gomerana*	Vězda; Lich. Sel. Exsic. 71 (DUKE)[g]	AF356684	Vězda; Lich. Sel. Exsic. 71 (DUKE)[g]	AF356685
	Xanthoria parietina	Søchting 7326 (C)[g]	AF356686	Søchting 7326 (C)[g]	AF356687
Pyrenomycetidae					
Diaporthales	*Diaporthe phaseolorum*	—	L36985	—	U47830
Hypocreales	*Hypocrea schweinitzii*	—	L36986	ATCC 90178[f]	AF279395
Xylariales	*Seynesia erumpens*	SMH 1291 (culture)[e]	AF279409	SMH 1291 (culture)[e]	AF279410
Loculoascomycetidae					
Dothideales	*Curvularia brachyspora*	—	L36995	ATCC 58872[f]	AF279380
Arthoniales	*Dendrographa minor*	R. Ornduff 10,070 (DUKE)	AF279381	R. Ornduff 10,070 (DUKE)	AF279382
	Schismatomma pericleum	—	U23540	Tehler 7701 (S)	AF279408

Lichinomycetidae					
	Lempholemma polyanthes	Zoladeski & Lutzoni 11294-L1(2/2) (CANL)	AF356690	Zoladeski & Lutzoni 11294-L1(2/2) (CANL)	AF356691
	Peltula obscurans	Lutzoni 96.10.26-101 1/2 (DUKE)	AF356692	Lutzoni 96.10.26-101 1/2 (DUKE)	AF356693
	Peltula umbilicata	Giess WG8680 1965-03-21 (COLO)	AF356688	Giess WG8680 1965-03-21 (COLO)	AF356689
Inoperculate discomycetes					
Helotiales	*Cudonia circinans*	—	AF107343	JP 232 (DNA)[d]	AF279379
	Fabrella tsuga	—	AF106015	JP 256 (DNA)[d]	AF356694
Rhytismatales	*Rhytisma acerinum*	JP (DUKE)[h]	AF356695	JP (DUKE)[h]	AF356696
Operculate discomycetes					
Pezizales	*Morchella esculenta*	—	U42642	ATCC 10968[f]	AF279398
	Urnula hiemalis	—	Z49754	—	Z48319 & Z248320
Hemiascomycetes					
Saccharomycetales	*Candida albicans*	—	X53497	—	X70659
	Saccharomyces cerevisiae	—	J01353	—	J01355
Archiascomycetes					
Neolectales	*Neolecta irregularis*	—	Z47721	JP176 (DUKE)[d]	AF279401
Schizosaccharomycetales	*Schizosaccharomyces pombe*	—	X54866	—	Z19136
Taphrinales	*Taphrina pruni*	—	AB000956	—	Z49792 & Z49793
Basidiomycota (outgroup)					
	Athelia bombacina	—	M55638	ATCC 20629[f]	AF279377
	Coprinus cinereus	—	M92991	—	AF041494

Note. New sequences (with sources of DNA) generated by this study that were deposited in GenBank are shown in boldface. GenBank accession numbers with source information that are not in bold were submitted to GenBank by FL as part of two previous studies (Bhattacharya et al., 2000; Lutzoni et al., 2001). GenBank accession numbers without source information correspond to sequences obtained from GenBank.

[a] When the name of the collector, the collection number, and the herbarium acronym (in parentheses) are provided, this means that the source of DNA was from herbarium specimens or fresh material now deposited at the specified herbarium.

[b] Dry specimen provided by Robert Lücking (Lehrstuhl für Pflanzensystematik, Universität Bayruth, 95447 Bayruth, Germany).

[c] Sequence provided by Imke Schmitt (Fachbereich 9/Botanik, Universität Essen, 45117 Essen, Germany).

[d] DNA corresponds to DNA aliquots provided by Jamie Platt (Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA).

[e] SMH corresponds to fungal cultures provided by Sabine Huhndorf (Department of Botany, The Field Museum of Natural History, Chicago, USA).

[f] ATCC corresponds to fungal cultures obtained from the American Type Culture Collection.

[g] Sequences provided by Ulrik Søchting (Botanical Institute, Department of Mycology, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen K, Denmark).

[h] Dry specimen provided by Jamie Platt (Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA).

Table 2
Primers used for symmetric PCR amplification and cycle sequencing reactions

Targeted region	Primer name[a]	Sequence	Position and orientation[b]	Reference
SSU rDNA	NS17	5′-CATGTCTAAGTTTAAGCAA-3′	54–72	Gargas and Taylor (1992)
	nssu97a	5′-TATACGGTGAAACTGCGAATGGC-3′	75–97	This study (F. Lutzoni)
	nssu97b	5′-CGGTGAAACTGCGAATGGC-3′	79–97	This study (F. Lutzoni)
	nssu131	5′-CAGTTATCGTTTATTTGATAGTACC-3′	107–131	This study (F. Lutzoni)
	SR11R	5′-GGAGCCTGAGAAACGGCTAC-3′	389–408	Spatafora et al. (1995)
	SR7	5′-GTTCAACTACGAGCTTTTTAA-3′	637–617	R. Vilgalys web site[c]
	SR7R	5′-TTAAAAAGCTCGTAGTTGAAC-3′	617–637	V. Vilgalys web site[c]
	nssu634	5′-CCCCAGAAGGAAAGICCCGICC-3′	705–684	This study (V. Reeb & F. Lutzoni)
	NS21	5′-GAATAATAGAATAGGACG-3′	802–819	Gargas and Taylor (1992)
	nssu897R	5′-AGAGGTGAAATTCTTGGA-3′	897–914	This study (V. Reeb & F. Lutzoni)
	nssu1088	5′-TGATTTCTCGTAAGGTGCCG-3′	1088–1069	This study (V. Reeb & F. Lutzoni)
	nssu1088R	5′-CGGCACCTTACGAGAAATCA-3′	1069–1088	This study (V. Reeb & F. Lutzoni)
	NS4	5′-CTTCCGTCAATTCCTTTAAG-3′	1150–1131	White et al. (1990)
	NS23	5′-GACTCAACACGGGGAAACTC-3′	1184–1203	Gargas and Taylor (1992)
	NS22	5′-AATTAAGCAGACAAATCACT-3′	1312–1297	Gargas and Taylor (1992)
	NS24	5′-AAACCTTGTTACGACTTTTA-3′	1769–1750	Gargas and Taylor (1992)
LSU rDNA	LR0R	5′-GTACCCGCTGAACTTAAG-3′	24–42	Rehner and Samuels (1994)
	LIC15R	5′-GGAGGAAAAGAAACCAACAG-3′	55–74	Miadlikowska et al. (2001)
	LIC24R	5′-GAAACCAACAGGGATTG-3′	64–80	Miadlikowska and Lutzoni (2000)
	LR3	5′-GGTCCGTGTTTCAAGAC-3′	677–661	Vilgalys and Hester (1990)
	LR3R	5′-GTCTTGAAACACGGACC-3′	664–680	R. Vilgalys web site[c]
	LR5	5′-ATCCTGAGGGAAACTTC-3′	997–981	Vilgalys and Hester (1990)
	LR5R	5′-GAAGTTTCCCTCAGGAT-3′	981–997	R. Vilgalys web site[c]
	LR6	5′-CGCCAGTTCTGCTTACC-3′	1173–1157	Vilgalys and Hester (1990)
	LIC2044	5′-ACGCCTGCCTACTCGCC-3′	1412–1396	This study (V. Reeb & F. Lutzoni)
	LR7	5′-TACTACCACCAAGATCT-3′	1483–1467	Vilgalys and Hester (1990)

[a] Map for all primers is available at Department of Biology, Duke University; http://www.morag.com/lutzoni/primer.shtml.
[b] Position of the primers relative to *Saccharomyces cerevisiae* (SSU rDNA, Mankin et al., 1986) and *Schizosaccharomyces pombe* (LSU rDNA, Lapeyre et al., 1993).
[c] Department of Biology, Duke University; http://www.biology.duke.edu/fungi/mycolab.

1 GHz, one dual PIII/933 MHz) running under Linux, three Macintosh computers (PowerPC 4400/200 MHz, PowerBook G3/500 MHz, PowerPC G4/400 MHz), the High Performance Parallel Computer Systems of the University of Kaiserslautern (equipped with a total of 50 MISP R10000 processors at 195–250 MHz), and the High Performance Computer Cluster facility at the Field Museum (6 dual Alpha nodes/500 MHz and 4 Ultra-SparcII/400 MHz). Maximum Parsimony and Maximum Likelihood analyses were carried out using PAUP* 4b4, 4b5, 4b6, 4b7, and 4b8 (Swofford, 1998–2001); all results obtained with PAUP*4b5 have been checked for accuracy prior to publication. Bayesian inference of phylogeny was done with MrBayes v1.11 (Huelsenbeck, 2000).

For a number of specimens we were unable to generate both SSU and LSU sequences, and in other instances, only the sequencing of the LSU was successful. For this reason we decided to analyze the data in two ways, based on two different sets of alignments: (1) combined phylogenetic analyses for all (62) taxa for which LSU and SSU rDNA sequences were available (ML1 and MP1) and (2) phylogenetic analyses (28 taxa) of LSU rDNA sequences, focusing on the Gyalectales and Ostropales using several outgroup taxa (chosen in accordance with the results from the combined ML1 and MP1 analyses) and including all taxa for which only LSU sequences were available (ML2 and MP2). Both alignments are available through the internet at http://www.morag.com/lutzoni/index.shtml.

2.4. Separate and combined analyses of the SSU and LSU data sets: 62 taxa

2.4.1. Test for combinability

We used a Bayesian approach to test if the LSU and SSU partitions were combinable. Each partition was first analyzed separately using MrBayes. The likelihood model and initial parameters were estimated using the Hierarchical Likelihood Ratio Test of the computer program Modeltest 3.04 (Posada and Crandall, 1998). The Markov chain Monte Carlo algorithm of MrBayes ran with five chains simultaneously, each initiated with a random tree, for 1,000,000 generations, sampling every 10th generation for a total of 100,000 trees sampled. The first 4500 sampled trees were discarded before calculating the majority-rule consensus tree to ensure that all chains had converged at a single level. A majority-rule

consensus tree was calculated with PAUP* for the remaining 95,500 B/MCMC sampled trees. The probability of each topological bi-partition was estimated by the frequency of these bi-partitions across all 95,500 trees sampled from the posterior probability distribution. A conflict was assumed to be significant if two different relationships for the same set of taxa (one being monophyletic and the other being non-monophyletic) both with posterior probabilities $\geqslant 99\%$ were observed on the SSU and LSU majority-rule consensus trees. Only if no significant conflict was detected throughout the majority-rule consensus trees, using this criterion, would the two partitions be combined.

2.4.2. Maximum Likelihood (ML1) analyses

An alignment of 62 taxa was used to estimate the position and test the monophyly of the Gyalectales and Ostropales within the ascomycetes. Alignments were manually optimized using Sequencher 3.0 (Gene Codes) for Macintosh and BioEdit 5.0.6 (Hall, 1999) for Microsoft Windows 95/98. Using the Hierarchical Likelihood Ratio Test implemented in Modeltest 3.04 (Posada and Crandall, 1998) the TrN three parameter nucleotide substitution model (Tamura and Nei, 1993) with equal base frequencies and a gamma shape distribution was selected. The ML search was conducted using PAUP* with the following parameter setting. The number of different rate categories was set to 2 (using three categories barely improved the likelihood score). The search was performed with 110 random-addition-sequence replicates (RAS), TBR swapping, and MULTREES in effect. Constant characters were excluded. Topological rearrangements were limited to 25,000 per replicate, which resulted (based on pre-run optimization of the heuristic search strategy) in a considerable reduction of the computing time without decreasing the accuracy of the search.

The level of confidence in the resulting topological bi-partitions was estimated with 552 bootstrap replicates and 3 RAS per bootstrap replicate. The number of RAS per bootstrap replicate was calculated by taking into account the number of times the shortest tree was found during the heuristic search, i.e., by at least doubling the number of RAS that were necessary to hit the optimal tree with a probability >99%. The statistical significance of the topological bi-partitions was tested with B/MCMC (Larget and Simon, 1999) as implemented in MrBayes v1.11 (Huelsenbeck, 2000). The same likelihood parameters as in the ML search were implemented. The sampling of trees using MrBayes was as described in the section "Test for combinability" above. The probability of each topological bi-partition was estimated by the frequency of these bi-partitions across all 95,500 trees sampled from the posterior probability distribution and are reported on the most likely tree derived from a heuristic search using ML as the optimization criterion. Clades with probabilities >95% were considered statistically significant.

2.4.3. Maximum Parsimony (MP1) analyses

The same combined data set for 62 taxa subjected to ML1 analyses was used for MP1 analyses with the exception that ambiguous regions were coded (INAASE 2.2b, Lutzoni et al., 2000) and unambiguously aligned gaps were not treated as missing data. The LSU and SSU data sets (unambiguously aligned portions) were subjected to a specific symmetric step matrix whose values were estimated by summarizing the observed frequency of changes between all possible character states (four nucleotide types plus gaps treated as fifth character state); these two frequency matrices were then converted to matrices of costs of changes using the negative natural logarithm (Felsenstein, 1981; Wheeler, 1990). All necessary calculations for step matrices were carried out using the character status function of PAUP* and a computer program written by F.K. Regions from the SSU and LSU that could not be unambiguously aligned due to sequence length variation among sequences were excluded, coded, and subjected to specific step matrices using the program INAASE 2.2b (Lutzoni et al., 2000), resulting in an additional 19 characters and step matrices for the MP analyses. None of the introns were coded into additional characters. The heuristic search for the best tree was performed with 1000 RAS, TBR swapping, MULTREES in effect, and using gaps as a fifth character state. Bootstrap support (Felsenstein, 1985) was estimated with 1103 replicates and 10 RAS per bootstrap replicate. The number of RAS per bootstrap replicate was calculated as described for ML1 above.

2.5. Phylogenetic analyses of the LSU data set for the Gyalectales–Ostropales complex: 28 taxa

2.5.1. Maximum Likelihood (ML2) analyses

Following Modeltest, we implemented the same likelihood model as in the combined ML1 analyses, i.e., TrN with gamma shape distribution and the number of different rate categories set to a value of 2, but with unequal base frequencies. The search was performed with 1000 RAS, TBR swapping, and MULTREES in effect, but without any rearrangement limits. Bootstrap support values were estimated with 1006 replicates, with 5 RAS per bootstrap replicate. The number of RAS per bootstrap replicate was calculated as described for ML1. The statistical significance of clades was also estimated using the B/MCMC method with the same model and parameters used for the ML2 search.

2.5.2. Maximum Parsimony (MP2) analyses

As for MP1 analyses, unequally weighted parsimony taking into account the frequency of nucleotide changes

was applied to this data set and INAASE was used to integrate phylogenetic signal from ambiguously aligned regions into these phylogenetic analyses without violating positional homology (Lutzoni et al., 2000). The search settings were the same as that for MP1 heuristic search. Bootstrap support was estimated with 1000 replicates, with 5 RAS per bootstrap replicate. The number of RAS per bootstrap replicate was calculated as described for ML1 analyses.

3. Results

3.1. DNA isolation, amplification, sequencing, and alignments

Fresh (collected no more than one year prior to DNA isolation) or deep-frozen material was essential for successful DNA isolation and amplification. When herbarium specimens were processed, the quality of the isolated DNA and PCR products was often low, sometimes resulting in unusable sequencing results.

The final length of the combined alignment for 62 taxa was 7601 sites, of which 3942 sites were from the SSU and 3659 sites were from the LSU. For the SSU, 199 sites from 13 ambiguous regions and 2727 sites from 15 introns were excluded from the analyses. In the ML analyses, gaps were treated as missing data, and the exclusion of 628 constant characters resulted in a total of 388 SSU characters used in ML1 analyses. With gaps treated as fifth character state, a total of 602 constant characters were excluded from the MP1 analyses, for a total of 414 SSU variable characters from which 225 were parsimony-informative. Ten of the 13 ambiguous regions could be coded and subjected to step matrices, using up to 32 different character states (the maximum number of character states that can be used by PAUP* on 32-bit Unix-based computers), resulting in 10 parsimony-informative characters for a total of 424 characters for MP1 analyses from which 235 were parsimony-informative characters for MP1 analyses. For the LSU, 389 sites from 19 ambiguous regions and 2320 sites from 20 introns were excluded. Treating gaps as missing data in ML analyses led to the exclusion of 558 constant characters, resulting in 392 LSU characters that were subjected to ML1 analyses. For MP1, a total of 540 constant characters were excluded from the LSU alignment with gaps treated as a fifth character state, resulting in 410 LSU variable characters from which 296 were parsimony-informative. The coding of LSU ambiguous regions resulted in nine additional parsimony-informative characters for the MP1 search for a total of 419 variable characters from which 305 were parsimony-informative. In total, 780 characters were used for the ML1 analyses and 540 parsimony-informative characters were used for the MP1 analyses.

The length of the alignment for the LSU data set with 28 taxa for the phylogenetic study focusing on the Gyalectales–Ostropales complex was 3486 sites in length. A total of 2159 sites from 17 introns were excluded from this second set of analyses, as well as 355 sites in 17 ambiguous regions. With gaps interpreted as missing data, 662 characters were constant, leading to 310 characters subjected to ML2 analyses. With gaps treated as an additional character state, 650 characters were constant and 187 of the remaining 322 characters were parsimony-informative. For the MP2 analyses all ambiguous regions were coded, leading to 17 additional characters for a total of 204 LSU parsimony-informative characters.

3.2. Combining SSU and LSU data sets: 62 taxa

The majority-rule consensus of the trees sampled with B/MCMC for the SSU and the LSU rDNA data sets, respectively, exhibited—although similar in their overall topology—various differences (data not shown). However, none of the different relationships revealed by the separate analyses received reciprocal posterior probabilities $\geqslant 99\%$, and therefore, combining these two data sets, was not considered to have any detrimental effect in estimating phylogenetic relationships among these taxa (Cunningham, 1997). Only in two cases (*Pyrenula cruenta* and *Rhytisma acerinum*) was the internodal probability of conflicting clades $\geqslant 95\%$. Based on SSU evidence, *R. acerinum* was nested within the operculate discomycetes (represented by *Morchella esculenta* and *Urnula hiemalis*) with a posterior probability of 98%, whereas with the LSU and combined analysis, this species was nested within the inoperculate discomycetes, both with posterior probabilities of 100%. The B/MCMC analysis of the LSU puts *Pyrenula cruenta* within the Lichinales (*Peltula obscurata*, *P. umbilicata*, and *Lempholemma polyanthes*) in 95% of all generated trees, whereas it was grouped with members of the Verrucariales and Chaetothyriales with the SSU analysis (99% probability for the clade containing both orders and *P. cruenta*) and in the combined analysis (100% posterior probability).

Testing for incongruence between data sets using MCMC algorithms and Bayesian statistics has been carried out here for the first time. Simulations are needed to get a more accurate estimation of the probability level at which combining conflicting data sets is more likely to converge on the wrong topology and conclusions. As indicated by Cunningham (1997), threshold values differ among various incongruence tests when used to determine if data sets should be combined, and therefore, cannot be generalized. In our study, both potentially problematic taxa (*Rhytisma acerinum* and *Pyrenula cruenta*) were, as supported by the morphological evidence and based on previous phylogenetic

studies (Bhattacharya et al., 2000; Lutzoni et al., 2001), correctly placed in the combined tree (Figs. 1 and 2), suggesting that our selection of 99% as the threshold to determine if data sets should be combined was appropriate.

3.3. Combined analyses (ML1 and MP1) of the SSU and LSU data sets for the Gyalectales and Ostropales within the Ascomycota: 62 taxa

3.3.1. Maximum Likelihood (ML1) analyses

The ML search revealed one most likely tree (Fig. 1, ln likelihood = −15074.51806), which was hit 87 times in 110 replicates (79.1%). The topology of the most likely tree was identical to the majority-rule consensus tree of 95,500 trees sampled with B/MCMC (not shown).

The same major groups of Ascomycota outlined by Lutzoni et al. (2001) were recovered here and their statistical significance was maintained or increased for most of them: the Euascomycetes with 100% posterior probability (99% BS), the next internode after the split of the Operculate Discomycetes has a posterior probability of 100%, the Lecanoromycetes (corresponding to node 6, "Lecanoromycotina," in Lutzoni et al., 2001) with 97% posterior probability, Unitunicate Ascohymenials ($P = 100\%$), Bitunicate Ascohymenials ($P = 96\%$), and the Loculoascomycetes with Pyrenomycetes clade ($P = 100\%$). The posterior probability for the Eurotiomycetidae dropped from 97 to 83%. Except for the Euascomycetes, the bootstrap frequencies for all the major groups listed above were below 70%, whereas most of these clades are statistically significant ($P > 95\%$) when using the B/MCMC. Preliminary results from an ongoing simulation study (Alfaro et al., in press) indicate that bootstrap is more likely than B/MCMC to fail to provide high support for correct short internodes.

The large order Lecanorales is paraphyletic, but forms a statistically significant monophyletic group ($P = 98\%$) with members of the Peltigerales, Caliciales, and Teloschistales (Table 1, Fig. 1). However, the exact relationships within this monophyletic group are still uncertain. The same is true for some early divergences within the Euascomycetes. More characters are needed to increase the low level of confidence in the deep branching patterns of the Euascomycetes as well as within the Bitunicate Ascohymenials, Unitunicate Ascohymenials, and Eurotiomycetidae.

All taxa belonging to the Gyalectales and Ostropales group together within the Unitunicate Ascohymenials, closely related to the Trapeliaceae ($P = 100\%$). The Gyalectales did not form a monophyletic group, because *Diploschistes* and *Graphina* were found to be sister to *Coenogonium*, *Dimerella*, and *Petractis luetkemuelleri* (Fig. 1). Together these taxa form a monophyletic group sister to *Gyalecta*, *Petractis hypoleuca*, and *P. clausa*. Most of the nodes in the Ostropales–Gyalectales were highly significant (posterior probabilities 95–100%). Many of the internal nodes exhibited very low bootstrap support (below 50%). Relationships among *P. clausa*, *Bryophagus*, and *Stictis* remain uncertain with both measures of confidence, as their affiliation to the remaining taxa. The latter two species were found to be outside the core Gyalectales–Ostropales clade ($P = 100\%$).

3.3.2. Maximum Parsimony (MP1) analyses

The MP search resulted in one most parsimonious tree (5,884.17 steps) that was hit 211 times out of 1000 (21.1%) RAS (Fig. 2). The most parsimonious tree differs slightly from the most likely tree resulting from the ML1 analysis, but none of these discrepancies received high support. Contrary to the ML1 tree, the Lichinomycetidae are sister to Loculoascomycetes and Pyrenomycetes. The Eurotiales (Fig. 2) are sister to the Pertusariales and Icmadophilaceae and are nested within the Bitunicate Ascohymenials instead of within the Eurotiomycetidae in the ML1 tree (Fig. 1), reflecting the weakly supported monophyly of the Eurotiomycetidae in the likelihood analysis. Additionally, the Unitunicate Ascohymenials–Acarosporaceae clade is now sister to the Bitunicate Ascohymenials–Eurotiomycetidae clade. Within the Gyalectales–Ostropales group, *Coenogonium*, *Dimerella*, *Petractis luetkemuelleri*, and *P. clausa* are sister to the *Gyalecta–Petractis hypoleuca* clade, and both are sister to the *Diploschistes–Graphina–Bryophagus* clade. All these discrepancies between MP1 and ML1 are in parts of the Ascomycota tree where relationships have always been uncertain (Bhattacharya et al., 2000; Lutzoni et al., 2001).

3.3.3. Bootstrap versus B/MCMC comparison for ML1 and MP1

The number of branches supported by bootstrap (26 internal branches with support $\geqslant 70\%$ in ML1 and 27 supported branches in MP1) is about two-thirds (65.0% [ML1] and 67.5% [MP1]) of the number of branches found to be statistically significant with B/MCMC (40 supported internal nodes in ML1, $P \geqslant 95\%$). In this case, MP bootstrapping with additional characters from coded ambiguous regions was equally efficient as an ML bootstrap. This substantial difference between bootstrap and B/MCMC might indicate that bootstrap has less statistical power and is biased toward providing very low support for short internodes, even if they are stable when using different taxon sampling and optimization criteria (cf. Bhattacharya et al., 2000; Lutzoni et al., 2001). This discrepancy between these two measures of confidence seems to increase as more taxa are included in the phylogenetic analysis (compare panels A and B of Fig. 3). As shown in Fig. 3, levels of confidence derived from the bootstrap or B/MCMC are dependent, to

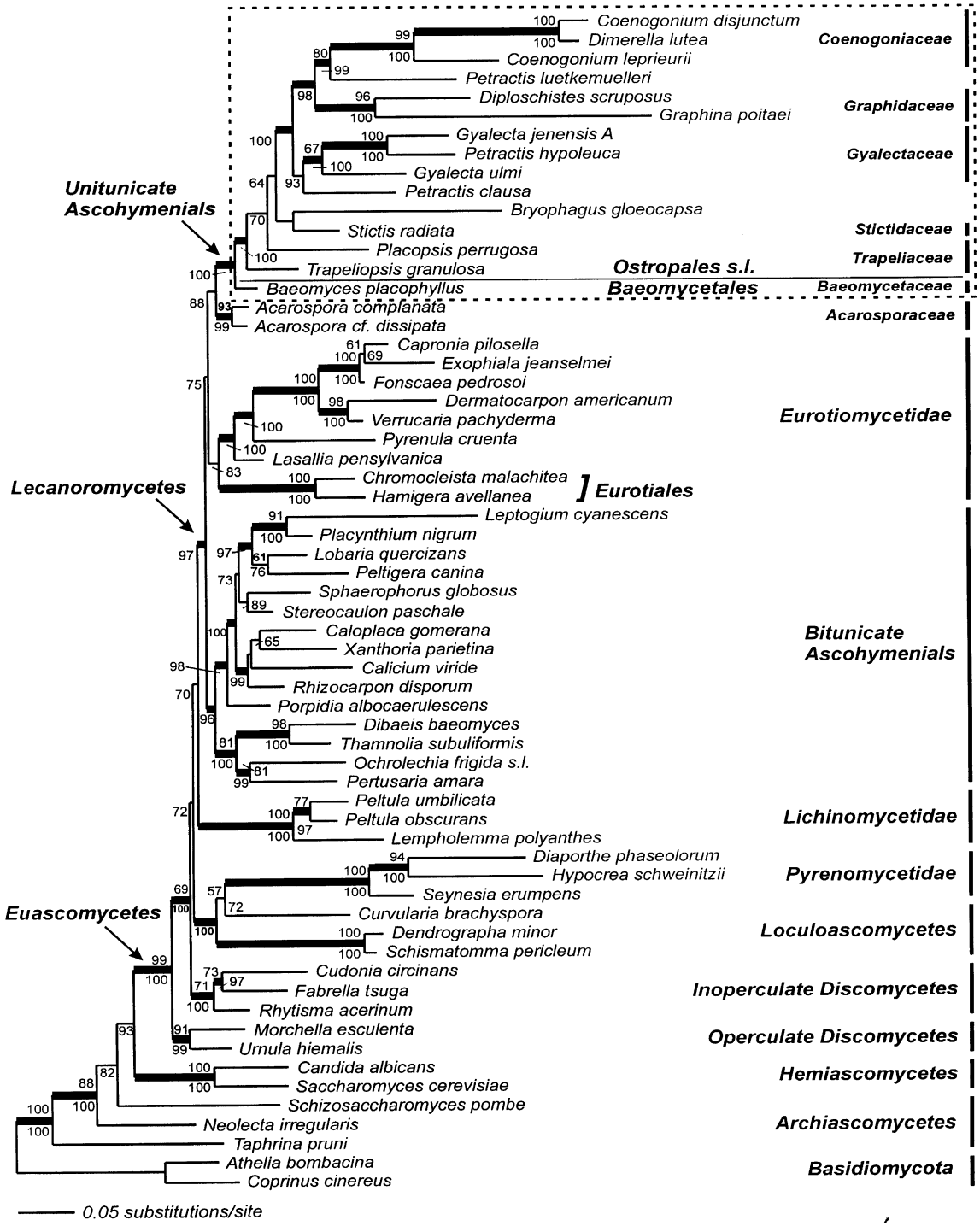

Fig. 1. ML1 analyses. Single most likely tree (ln likelihood = −15074.51806) resulting from the combined phylogenetic analysis of the nuclear SSU and LSU rDNA for 60 species belonging to the Ascomycota and two basidiomycete outgroup species. Numbers above internodes are bootstrap support values (%) and values below internal branches are B/MCMC posterior probabilities (%). For both estimates of support, only values above 50% are shown. If either the likelihood bootstrap support is $\geqslant$ 70% or the bayesian posterior probabilities $\geqslant$ 95%, the internal branch is shown as a thicker line.

various degrees, on branch lengths. Because bootstrap values result from resampling characters multiple times, it might be virtually impossible for this method to provide high support for short internodes, even if they are accurate (Berbee et al., 2000; Alfaro et al., in prep.). In the ML1 bootstrap (Fig. 3A), all internodes with relative branch lengths $\geqslant$ 12.5% (relative to the maximum branch length observed) exhibit support $\geqslant$ 70% and no

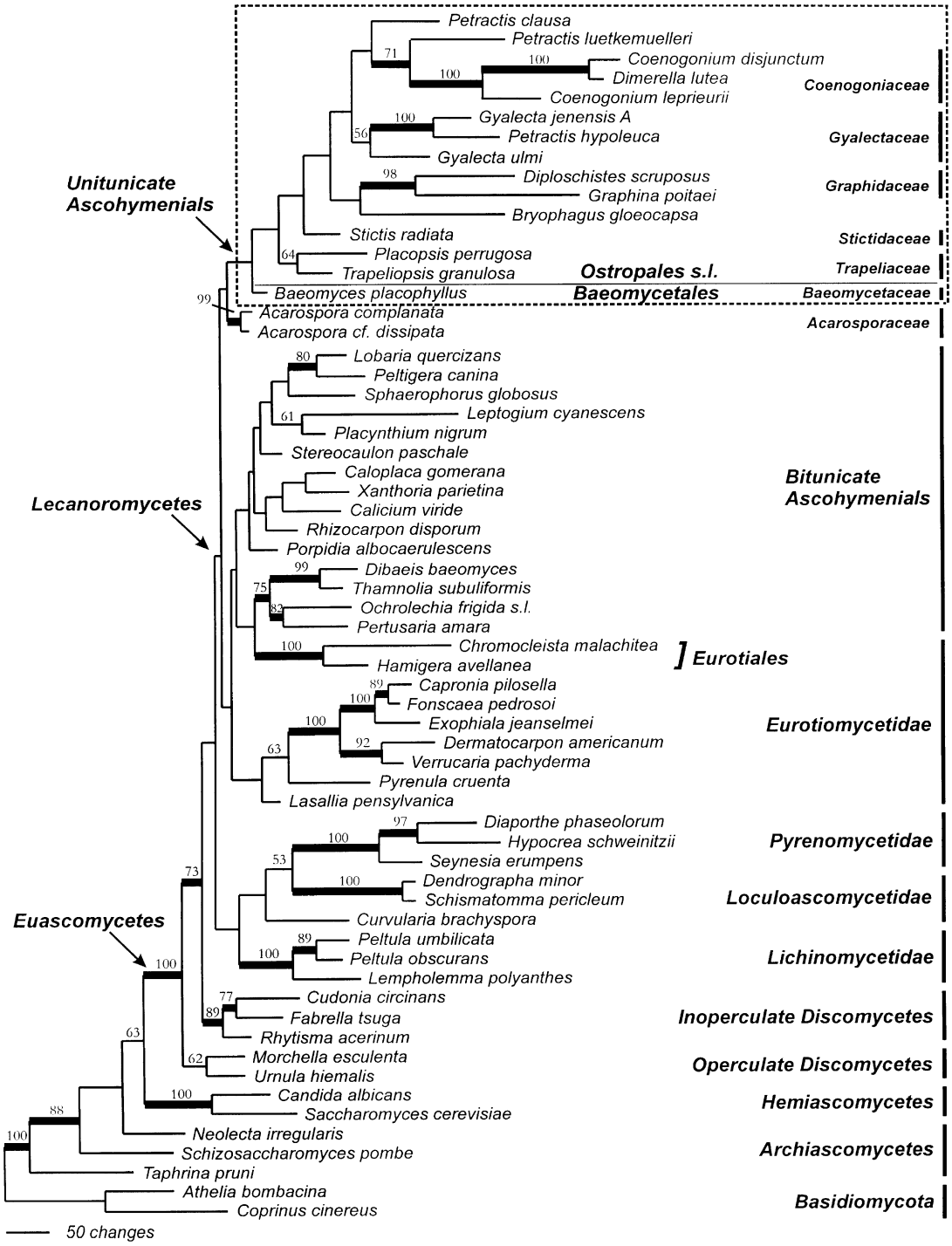

Fig. 2. MP1 analyses. Single most parsimoniuos tree (5884.17 steps, CI = 0.4710, CI excluding uninformative characters = 0.4023, RI = 0.4968), resulting from the combined phylogenetic analysis of the SSU and LSU rDNA for 60 species belonging to the Ascomycota and two basidiomycete outgroup species. Bootstrap values are shown above internal branches when $\geqslant 50\%$.

internodes with relative branch lengths <5% had bootstrap values $\geqslant 70\%$. The minimum relative branch length associated with bootstrap values $\geqslant 70\%$ in the MP1 bootstrap was 10%, and all branches with a relative length $\geqslant 24\%$ had bootstrap support $\geqslant 70\%$. In the B/MCMC analysis, the threshold relative branch length beyond which all internodes were significant ($P \geqslant 95\%$) was 12.5%, and the minimum relative branch length at which an internode was found significant was around 2.5%.

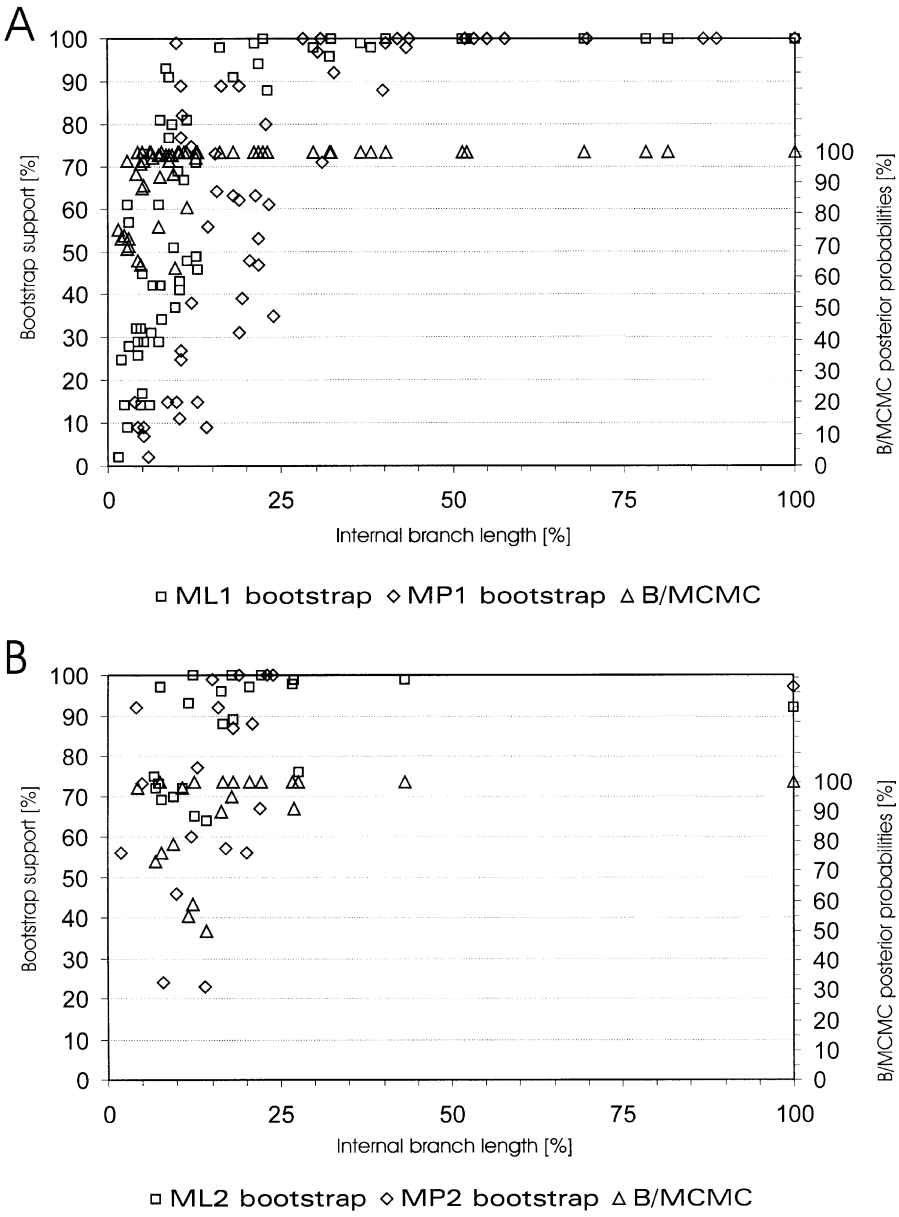

Fig. 3. Comparison of internode support with branch length. Internode support relative to length of internal branches estimated with bootstrap and Bayesian inference/Markov chain Monte Carlo. The threshold for significant B/MCMC posterior probabilities ($P = 95\%$; right Y axis) is at the same level as 70% bootstrap support (left Y axis). (A) ML1 and MP1 analyses of combined nuclear SSU and LSU rDNA data sets for 62 taxa. (B) ML2 and MP2 analyses of nuclear LSU rDNA data set for 28 taxa.

The number of internal branches supported with B/MCMC probabilities $\geqslant 95\%$ decreases from 40 supported branches in the combined analysis to 19 with the SSU and 31 with the LSU when analyzed separately. This comparison emphasizes not only the low potential of the SSU data set alone to provide significant level of confidence for bi-partitions, but also the substantial increase in confidence level we gain when combining the two data sets. Therefore, this major decrease in uncertainty resulting from our combined SSU and LSU using B/MCMC as presented in Lutzoni et al. (2001) when compared to trees based solely on SSU or LSU is not only due to the use of a potentially more powerful approach (B/MCMC) to estimate confidence levels, but is also the result of combining these two data sets. Prior to this study, only three broad phylogenetic studies of the Ascomycota were based on the fusion of two data sets (Bhattacharya et al., 2000; Lutzoni et al., 2001; Suh and Blackwell, 1999). The addition of more characters still has a major impact in decreasing the level of uncertainty associated with broad phylogenies of the Ascomycota when using B/MCMC.

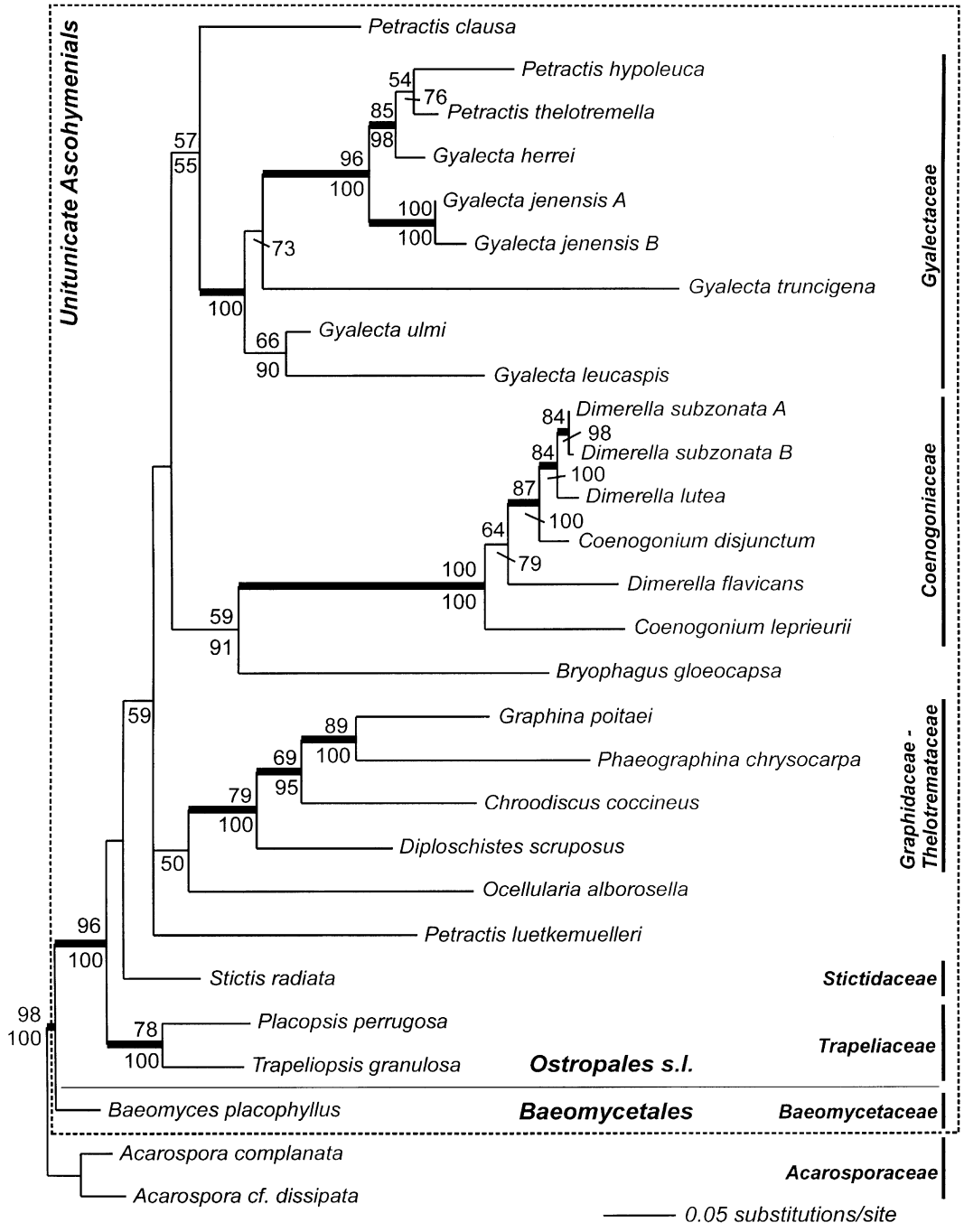

Fig. 4. ML2 analyses. One of the three equally most likely trees (ln likelihood = −3603.27921), identical to the strict consensus tree for these three trees, based on LSU rDNA data alone for 26 species belonging to the Unitunicate Ascohymenials and two outgroup *Acarospora* species. Bootstrap values and posterior probabilities ⩾ 50% are shown above and below internal branches, respectively.

3.4. Phylogenetic analyses of the nuclear LSU rDNA for the Gyalectales–Ostropales complex: 28 taxa

3.4.1. Maximum Likelihood (ML2)

This ML search resulted in three equally most likely trees (ln likelihood = −3603.27921), which were hit 403 times out of 1000 RAS (40.3%). One of these three trees showed a polytomy and was identical to the strict consensus tree for these three trees. This unresolved phylogram that also depicts the strict-consensus tree is presented in Fig. 4. The strict-consensus tree was identical to the majority-rule consensus tree of 95,500 trees sampled with B/MCMC, with the exception that the latter had two additional polytomies: (1) posterior probabilities were <50% for relationships among the *Gyalecta* clade, the *Coenogonium* clade, the Thelotre-

mataceae–Graphidaceae clade, and *Petractis luetkemuelleri* with B/MCMC, resulting in a polytomy, and (2) *Stictis radiata* and the Trapeliaceae formed a trichotomy with this polytomic clade in the B/MCMC consensus tree.

As for the tree based on the combined SSU and LSU data, *Baeomyces* and the Trapeliaceae are close to the Acarosporaceae in the ML2 tree (Fig. 4). The same three main clades of the Ostropales–Gyalectales complex revealed by the ML1 combined analysis (Fig. 1) (i.e., *Dimerella–Coenogonium*, *Gyalecta-Petractis p.p.*, Graphidaceae–Thelotremataceae) are also present in the ML2 LSU tree (Fig. 4). *Petractis clausa* is sister to the *Gyaletca-Petractis p.p.* clade, and together, they are sister to the *Coenogonium–Dimerella* clade with *Bryophagus gloeocapsa* at its base. The relationship of these two major clades (*Coenogonum–Dimerella*, *Gyalecta–Petractis p.p.*) and their connection to the Thelotremataceae–Graphidaceae clade is unsupported by BS or B/MCMC. The affiliations of *Stictis radiata* and *Bryophagus gleocapsa* remain highly uncertain in this analysis, whereas the sister relationship of *Petractis luetkemuelleri* to the *Dimerella–Coenogonium* clade revealed by the ML1 combined analyses was not comfirmed by the ML2 analyses restricted to the Unitunicate Ascohymenials and the LSU. The sister relationship of *Ocellularia alborosella* to the Graphidaceae was associated with a low probability of 50%.

3.4.2. Maximum Parsimony (MP2)

This MP search revealed one most parsimonious tree (Fig. 5; 2,239.99 steps), which was found in 439 out of 1000 RAS (43.9%). This increase in resolution, compared to the ML2 search, is likely to result from the 19 additional INAASE characters that could be included in the MP2 analysis. The topology of the best MP2 tree differs from the strict consensus ML2 tree (Fig. 4) in several points; however, these differences affect the same taxa with uncertain relationships as revealed by the likelihood search. None of these differences is supported with a bootstrap higher than 60%. The main well-supported clades of the ML2 search were also found by the MP2 search, and as for ML2, their relationships lack support.

3.4.3. Bootstrap versus B/MCMC comparison for ML2 and MP2

Discrepancies between bootstrapping and B/MCMC for 28 taxa (Fig. 3B) are not as striking as in the combined analysis for 62 taxa. MP bootstrapping with additional characters from ambiguous regions (MP2) and ML bootstrapping (ML2) were equally efficient and exhibited 12 internal branches with support $\geqslant 70\%$. With B/MCMC, 14 internal branches were found to be statistically significant ($P \geqslant 95\%$). In the ML2 bootstrap, the minimum relative branch length with support $\geqslant 70\%$ is 7%, and all branches $\geqslant 15\%$ are supported at the $\geqslant 70\%$ level. In the MP2 bootstrap, no internal branch with a relative length $<4\%$ and all branches with a relative length $\geqslant 23\%$ are supported with bootstrap values $\geqslant 70\%$. Similar to ML2 and MP2, the minimum relative branch length for support ($P \geqslant 95\%$) in B/MCMC was 5%. The threshold relative branch length beyond which all internodes were found to be significant was 28%, the highest value in this comparison.

4. Discussion

4.1. Taxonomy

The term Unitunicate Ascohymenials was erected by Tehler (1996) to comprise all ascomycetes with an ascohymenial ascoma development (Nannfeldt, 1932) and unitunicate asci (Luttrell, 1951), that is, asci that are functionally one-layered. According to Tehler (1996) circumscription of this group, this is a paraphyletic assemblage defined by two symplesiomorphies: (1) unitunicate asci with an apical spore apparatus and (2) a hamathecium with true paraphyses. The Unitunicate Ascohymenials as delineated by Tehler (1996) differ from our recircumscription, by the inclusion of the Hypocreales and Helotiales (Leotiales). The Hypocreales have been shown by several independent molecular phylogenetic studies to be part of the Pyrenomycetes (Berbee, 1996; Bhattacharya et al., 2000; Lutzoni et al., 2001; Spatafora et al., 1995; Suh and Blackwell, 1999) and are confirmed here to be part of the Pyrenomycetidae (Figs. 1 and 2). In our study, *Baeomyces* is the only genus of the Helotiales (sensu Tehler) that is part of the Unitunicate Ascohymenials (with $P \geqslant 95\%$, Lutzoni et al., 2001, and this study) and therefore in agreement with Tehler's (1996) classification. The Icmadophilaceae are part of the Bitunicate Ascohymenials ($P = 96\%$) according to our study, although this sister relationship of the Pertusariales–Icmadophilaceae group to the rest of the Bitunicate Ascohymenials was not significant in Lutzoni et al. (2001), which could indicate instability resulting from taxon sampling for this specific relationship. Nevertheless, in both studies, the Icmadophilaceae are part of the Lecanoromycetes, i.e., outside of the Helotiales s. str. The Helotiales s. str., represented here by *Cudonia* and *Fabrella*, along with the Rhytismatales, are likely to be part of the second main divergence during the early evolution of the Euascomycetes (Lutzoni et al., 2001). The exclusion of the Baeomycetaceae and Icmadophilaceae from the Helotiales is in agreement with Platt and Spatafora (2000).

This study confirms that the Gyalectales and Ostropales, as currently delineated, are very closely related orders, disproving the suggestion that they are distantly

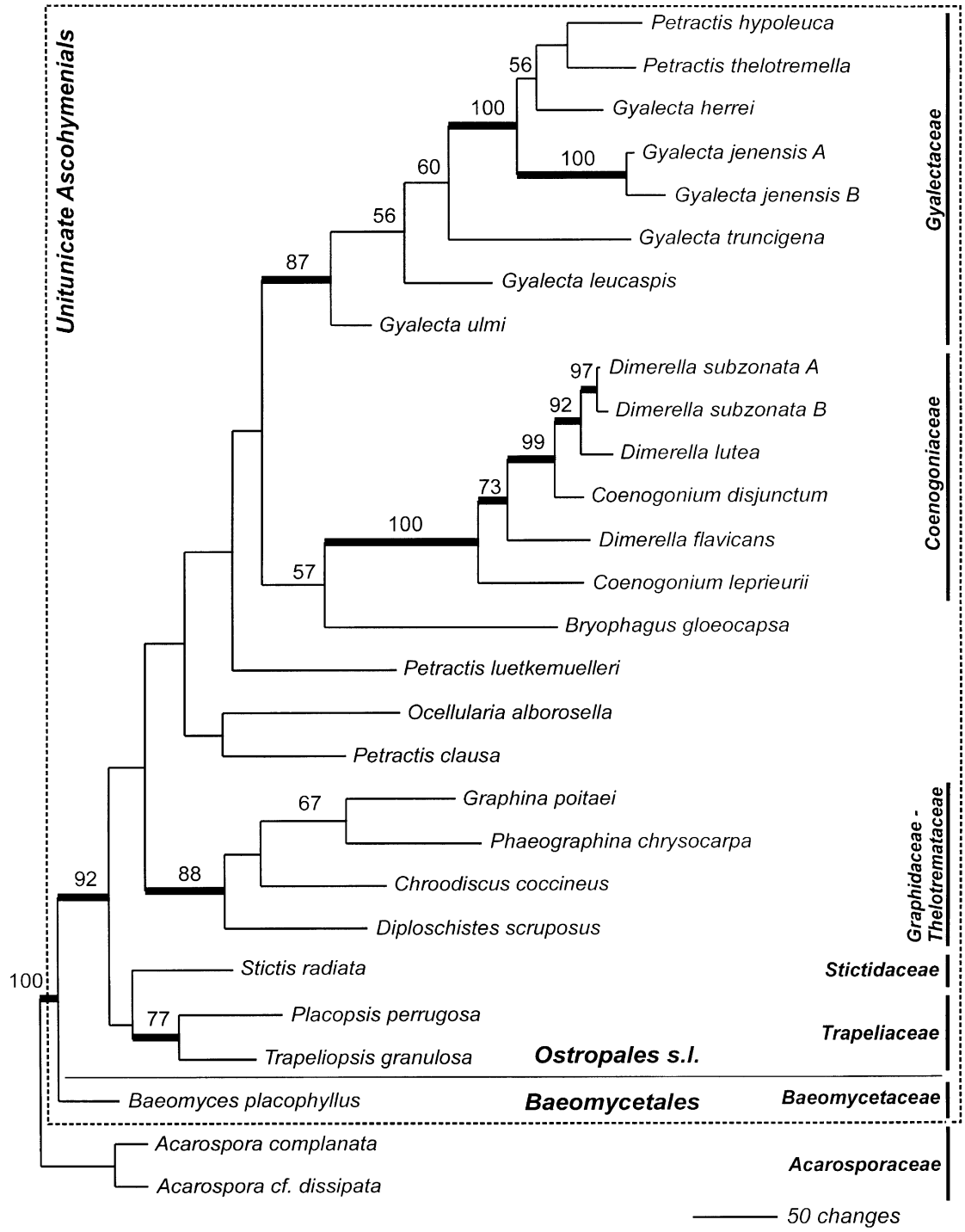

Fig. 5. MP2 analyses. Single most parsimonious tree (2239.99 steps, CI = 0.6725, CI excluding uninformative characters = 0.6261, RI = 0.6576) based on LSU rDNA data alone for 26 species belonging to the Unitunicate Ascohymenials and two outgroup *Acarospora* species. Bootstrap values $\geqslant$ 50% are shown above internal branches.

related because their numerous anatomical, morphological, and developmental similarities are homoplasious (Henssen and Jahns, 1974). In accordance with their ascus anatomy and ultrastructure (Henssen and Jahns, 1974), the Ostropales and Gyalectales are classified within the Unitunicate Ascohymenials. Their intermixed relationships revealed by our phylogenetic study mean that we can no longer treat these two orders as two separate monophyletic entities. Because the Gyalectales is the most recently named of the two orders (1932 for Ostropales vs. 1986 for Gyalectales), it must be subsumed within the Ostropales.

Given our taxon sampling, the closest relatives to the Ostropales s.l. (i.e., including Gyalectales) are the Trapeliaceae, but the exact phylogenetic placement of *Placopsis* and *Trapeliopsis* remains uncertain. *Stictis* is the *typus generis* of Stictidaceae and of the Ostropales. The close relationship between *Stictis* and the Trapeliaceae and the phylogenetic uncertainty within this family justify the inclusion of the Trapeliaceae into the Ostropales until stronger evidence shows that the Trapeliaceae, and the Agyrinae in general, form a monophyletic entity, sister to the Ostropales s.l. Lumbsch et al. (2001) raised the Agyrinae to the ordinal level as a sister group to the Ostropales. However, the bootstrap supports for the monophyly of the "Agyriales" (61%) and for the sister group relationship to Ostropales and Pertusariales (<50%) of their Maximum Parsimony-based analysis of SSU data are too low to justify this taxonomical decision. If left outside of the Ostropales, the uncertainty associated with the Trapeliaceae would result in an unstable phylogenetic classification within the Unitunicate Ascohymenials as relationships within the Trapeliaceae sway between para- and monophyletic assemblages (Figs. 1, 2, 4, and 5). The Trapeliaceae (as well as the Acarosporaceae) are currently listed in the Lecanorales (Eriksson and Hawksworth, 1998), a classification that can no longer be upheld. The Acarosporaceae are one of the four main lineages within the Lecanoromycotina (sensu Lutzoni et al., 2001), but their relationship to the other three lineages (Unitunicate Ascohymenials, Bitunicate Ascohymenials, and Eurotiomycetidae) remains uncertain (Lutzoni et al., 2001).

Based on our phylogenetic analyses of the combined SSU–LSU rDNA data set (62 taxa) and smaller LSU data set (28 taxa), four distinct monophyletic core entities can be distinguished (Fig. 4) with significant support within the Ostropales s.l. (including Gyalectales and Graphidaceae): (1) a *Gyalecta* clade ($P = 100\%$), including *Gyalecta*, *Petractis hypoleuca*, and *P. thelotremella*, (2) a *Coenogonium* clade ($P = 100\%$), including *Coenogonium* (with the former genus *Dimerella*; Lücking and Kalb, 2000), (3) a Graphidaceae–Thelotremataceae clade, represented by *Diploschistes*, *Chroodiscus*, *Phaeographina*, and *Graphina*, and (4) a Trapeliaceae clade, represented by *Placopsis* and *Trapeliopsis*. Relationships for all other taxa within the Ostropales s.l. cannot be estimated with sufficient support in any of the analyses performed here. However, based on the ML1 and ML2 trees (Figs. 1 and 4) *Gyalecta* and *Coenogonium* (as defined here) are not likely to be part of the same family, and for this reason, we kept these two genera as part of two different families: the Gyalectaceae and the Coenogoniaceae.

A single family containing all members of the former Gyalectaceae sensu Henssen and Jahns (1974) would also have to include genera nested in the Graphidaceae clade for not violating the monophyly of the family. Therefore, we propose here to classify *Diploschistes*, *Chroodiscus*, *Phaeographina*, and *Graphina* as part of the Graphidaceae and keep *Stictis radiata* as a representative of the Stictidaceae. The Unitunicate Ascohymenials would then consist of at least two orders, the Ostropales and the Baeomycetales, and the Ostropales would consist of at least five families, the Gyalectaceae, Coenogoniaceae, Graphidaceae, Stictidaceae, and Trapeliaceae. Due to our limited taxon sampling from the Graphidaceae–Thelotremataceae clade, it seems too early to further separate this group into two families (Graphidaceae and Thelotremataceae). The phylogenetic placement of *Petractis luetkemuelleri*, *P. clausa*, and *Ocellularia alborosella* is still uncertain in this provisional classification.

Coenogonium and *Dimerella* have always been considered to be two very closely related genera (Müller-Argoviensis, 1881). They were distinguished only by their growth form, which separates the filamentous lichen *Coenogonium* from its crustose counterpart *Dimerella*. The use of this single character for the delimitation of the two genera has always been questionable and the discovery of species exhibiting thallus characteristics of both *Dimerella* and *Coenogonium* eventually made their fusion unavoidable (Lücking and Kalb, 2000). However, new combinations have not been done for all *Dimerella* species. The results of our study clearly confirm the monophyly of the genus *Coenogonium* sensu Kalb and Lücking and a recent study by Kauff and Büdel (in prep.) focusing on ascoma ontogeny supports the separation of this genus from the remaining members of the Gyalectaceae. *P. thelotremella* and *P. hypoleuca* are undoubtedly nested within *Gyalecta* (Fig. 4, ML2: $P = 100\%$, $BS = 60\%$; Fig. 5, MP2: $BS = 88\%$) and need to return to *Gyalecta* in agreement with Zählbruckner (1924) as *Gyalecta thelotremella* Bagl. and *Gyalecta hypoleuca* (Ach.) Zahlbr.

In the ML1 tree (Fig. 1), the fourth species of *Petractis*, *P. clausa*, which is also the type species of this genus, is sister to *Gyalecta* with a Bayesian posterior probability of 93%, whereas its position remains completely unsupported in the MP1, MP2, and ML2 trees. In this context, the affiliation of the only other species of *Petractis*, *P. farlowii*, which, like *P. clausa*, bears cyanobacteria as symbionts, needs to be included in further phylogenetic studies with a more extensive taxon sampling and additional characters.

Our results imply several changes to the classifications proposed in the current "Outline of the Ascomycetes" (Eriksson and Hawksworth, 1998) and the eighth edition of the "Dictionary of the Fungi" (Hawksworth et al., 1995). All taxa currently listed in the order Gyalectales and its only family the Gyalectaceae are now subsumed under the Ostropales. The new family Coenogoniaceae, comprising *Coenogonium* s.l. (including *Dimerella*), is recognized here as a separate family from the Gyalectaceae, which includes the remaining genera *Gyalecta*,

Belonia, *Cryptolechia*, *Pachyphiale*, *Ramonia*, and *Semigyalecta*. The genus *Gyalecta* now also comprises two species formerly belonging to *Petractis*. The Trapeliaceae, formerly classified in the Lecanorales, are transferred to the Ostropales.

4.2. Internode support estimation

In this study, we used three different methods to estimate internode support: bootstrapped data sets analyzed with MP and ML and Bayesian inference/Markov chain Monte Carlo algorithms as available in the program MrBayes. The bootstrap and the Bayesian approaches show strong differences in estimating internode support, especially when a large number of taxa with multiple short internal branches are present. In ML1, internal branches with high bootstrap support (>70%) were always highly probable ($P \geqslant 97\%$). However, the reverse was often not true; of the 41 internal branches with probabilities $\geqslant 95\%$, only 26 showed bootstrap support $\geqslant 70\%$. When applied to the much smaller data set of 28 taxa for ML2 and MP2 (compared to 62 taxa for ML1 and MP1), all branches with bootstrap support $\geqslant 77\%$ showed posterior probabilities $P \geqslant 98\%$, but only one of the internal branches with $P \geqslant 95\%$ exhibited bootstrap support <70%, with a bootstrap value of 69%. Hence, in the MP2 and ML2 analyses of 28 taxa, the number of internal branches supported by B/MCMC does not exceed the number of branches supported by bootstrapping as much as it did in the combined analyses for 62 taxa. Bootstrap values have a tendency to decrease when the number of investigated taxa increases (Sanderson, 1995, 2000), which could explain why the number of supported internal branches is more similar when comparing ML2 and MP2 to B/MCMC analyses of the 28 taxa data set. It is therefore expected that large-scale phylogenetic studies will most benefit from the B/MCMC approach when estimating phylogenetic uncertainty. Such strong differences between B/MCMC and ML bootstrapping are especially surprising (Larget and Simon, 1999).

Although the results of the Bayesian approach and the standard ML search were similar in terms of phylogenies recovered by both methods, the required computing times were very different. For the combined analysis, it took MrBayes on a 1 GHz-Athlon PC about 28 h of computing time running five simultaneous MCMC chains for 1,000,000 generations. On the same machine, finding the optimal tree using PAUP* needs c. 287 h for the tree search (100 RAS) and c. 1200 h (about 50 days) for 100 bootstrap replicates.

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