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Performance of four ribosomal DNA regions to infer higher-level phylogenetic relationships of inoperculate euascomycetes (Leotiomyceta)

H. Thorsten Lumbsch[a,*], Imke Schmitt[a], Ralf Lindemuth[b], Andrew Miller[c], Armin Mangold[a,b], Fernando Fernandez[a], Sabine Huhndorf[a]

[a] *Department of Botany, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA*
[b] *Universität Duisburg-Essen, Campus Essen, 45117 Essen, Germany*
[c] *Center for Biodiversity, Illinois Natural History Survey, 607 E. Peabody Drive, Champaign, IL 61820, USA*

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Abstract

The inoperculate euascomycetes are filamentous fungi that form saprobic, parasitic, and symbiotic associations with a wide variety of animals, plants, cyanobacteria, and other fungi. The higher-level relationships of this economically important group have been unsettled for over 100 years. A data set of 55 species was assembled including sequence data from nuclear and mitochondrial small and large subunit rDNAs for each taxon; 83 new sequences were obtained for this study. Parsimony and Bayesian analyses were performed using the four-region data set and all 14 possible subpartitions of the data. The mitochondrial LSU rDNA was used for the first time in a higher-level phylogenetic study of ascomycetes and its use in concatenated analyses is supported. The classes that were recognized in Leotiomyceta (= inoperculate euascomycetes) in a classification by Eriksson and Winka [Myconet 1 (1997) 1] are strongly supported as monophyletic. The following classes formed strongly supported sister-groups: Arthoniomycetes and Dothideomycetes, Chaetothyriomycetes and Eurotiomycetes, and Leotiomycetes and Sordariomycetes. Nevertheless, the backbone of the euascomycete phylogeny remains poorly resolved. Bayesian posterior probabilities were always higher than maximum parsimony bootstrap values, but converged with an increase in gene partitions analyzed in concatenated analyses. Comparison of five recent higher-level phylogenetic studies in ascomycetes demonstrates a high degree of uncertainty in the relationships between classes.
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1. Introduction

The Ascomycota is the largest group of fungi (Kirk et al., 2001) and is characterized by the endogenous formation of spores in a sac-like meiosporangium, the so-called ascus. These fungi colonize a large variety of habitats and utilize a broad range of nutrient sources. Within the Ascomycota, the euascomycetes (= Pezizomycotina) is a group of filamentous fungi in which the asci are usually concentrated in a fruiting body of definite morphology, the ascoma. The euascomycetes include species that form saprobic, parasitic, and symbiotic associations with animals, plants (including algae, bryophytes, and phanerogams), other fungi, or cyanobacteria (Alexopoulos et al., 1996). This study deals with the euascomycetes that have inoperculate asci, which are classified in the superclass Leotiomyceta (Eriksson and Winka, 1997). They are distinguished from the basal Pezizomyceta, which include the apotheciate Pezizales with operculate asci.

* Corresponding author. Fax: +1 312 665 7158.
E-mail address: tlumbsch@fmnh.org (H.T. Lumbsch).

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The higher-level classification of inoperculate euascomycetes has been unstable for almost 100 years. Most classifications employed only single characters to distinguish major groups within euascomycetes and these were subsequently found to have limited phylogenetic information. In the classical period of the 19th and early 20th century, groups of euascomycetes were distinguished on the basis of morphology of the ascomata, resulting in a schematic distinction of classes, such as Discomycetes including species with apothecia, Plectomycetes with cleistothecia, and Pyrenomycetes with perithecia. This classification was recognized as too coarse (e.g., von Höhnel, 1907) and did not allow proper placement of taxa with intermediate ascoma-types. Consequently, other characters were used for the circumscription of major clades of eusacomyetes, such as the ascoma development (Nannfeldt, 1932) and ascus-type (Luttrell, 1955). However, these classifications were also based on single characters and became unstable with growing knowledge of the morphological diversity of these organisms. Conflicting classifications have been proposed for supraordinal categories in euascomycetes and subsequently Eriksson and Hawksworth (1993) avoided all supraordinal ranks in their classification of ascomycetes.

Molecular data have been employed for more than a decade in mycology to test morphology-based classifications that were shown to produce unnatural groupings when used as a single criterion. Phylogenetic studies employing nuclear SSU rDNA sequences provided support for a modified classification. It was shown that a combination of ascoma-types, ascoma development, and ascus-type was useful to circumscribe monophyletic clades, even though the individual characters were homoplasious (Berbee and Taylor, 1992, 1995; Gargas and Taylor, 1995; Lumbsch, 2000; Spatafora, 1995; Winka, 2000). These findings resulted in a new supraordinal classification including the distinction of several classes of closely related orders that was based on the combination of morphological characters and SSU rDNA molecular characters (Eriksson and Winka, 1997). However, some of the classes proposed by Eriksson and Winka (1997), such as Dothideomycetes or Lecanoromycetes, did not receive support in nuclear SSU rDNA phylogenies and the relationships among the classes remained unclear. Further, Tehler et al. (2000, 2003) conducted large-scale analyses of all nuclear SSU rDNA data then available from fungi, and showed that this data set alone is insufficient to resolve higher-level phylogeny of euascomycetes with confidence. Four additional molecular data sets have so far been added to the toolbox for the elucidation of the phylogeny of higher-level euascomycetes: the protein-coding genes β-tubulin (Landvik et al., 2001) and RPB-2 (Liu et al., 1999; Liu and Hall, 2004), the nuclear LSU rDNA (Bhattacharya et al., 2000; Lumbsch et al., 2000; Lutzoni et al., 2001), and the mitochondrial SSU rDNA (Lindemuth et al., 2001; Lumbsch et al., 2002). The analysis of the two additional ribosomal data sets basically confirmed the classification based on nuclear SSU rDNA. In concatenated analyses some poorly supported classes, such as Dothideomycetes and Lecanoromycetes, gained strong support, and some relationships among classes, such as the sister-group relationship of Chaetothyriomycetes and Eurotiomycetes, were strongly supported. However, most relationships between classes remained poorly supported. The analysis of β-tubulin sequences in ascomycetes is complicated due to the presence of paralogues (Landvik et al., 2001) and thus this data set was not further explored in higher-level phylogenetic studies in euascomycetes. The results of the RPB-2 analyses (Liu et al., 1999; Liu and Hall, 2004) supported the monophyly of most classes distinguished by Eriksson and Winka (1997), but the relationships between these classes differed from analyses based on ribosomal genes in that loculoascomycetes formed a monophyletic group. In a study including three large-scale analyses of combined data sets of (a) nuclear small and large subunit ribosomal DNA, and in addition (b) RPB-2, and (c) RPB-2 and mitochondrial small subunit ribosomal DNA sequences, Lutzoni et al. (2004) found most of the classes distinguished by Eriksson and Winka (1997) as monophyletic except the Dothideomycetes and Leotiomycetes that were para- or polyphyletic in some analyses. Several changes to the classification of Eriksson and Winka (1997) were accepted by Lutzoni et al. (2004). An additional class, the Lichinomycetes was accepted, which was described previously (Reeb et al., 2004). However, species in this class were nested within Dothideomycetes or a paraphyletic Leotiomycetes in the different analyses. No representative of the Lichinomycetes sensu Reeb et al. (2004) is included in our study, since we were unable to obtain mt LSU rDNA sequences from any species in this group. In the study by Lutzoni et al. (2004) the Arthoniomycetes and Dothideomycetes were classified as subclasses of Sordariomycetes, although they were paraphyletic in the analysis of the nuclear ribosomal DNA and only formed a monophyletic clade in the three and four gene analyses that included only one or two representatives of these two classes. The Chaetothyriomycetes and Eurotiomycetes were found as sister-groups in all three analyses and hence classified as subclasses within one class Eurotiomycetes sensu lato.

Given the uncertainty of the backbone of the euascomycete phylogeny, we have targeted the mitochondrial LSU rDNA as an additional data set to elucidate the higher-level phylogeny within inoperculate euascomycetes. This gene has rarely been used for phylogenetic studies in ascomycetes and mainly at intrageneric or intrafamiliar rank (e.g., Peever et al., 2004; Schmitt and Lumbsch, 2004). However, it has been employed at higher-level phylogenetic studies in basidiomycetes (e.g.,

Binder and Hibbett, 2002). Here we evaluate the use of the mitochondrial LSU rDNA for phylogenetic problems at higher ranks in euascomycetes.

To explore the influence of each data set in combined analyses, we performed separate analyses for each data set and combined analyses for every possible combination, resulting in 15 analyses. Recently, differences among posterior probabilities obtained in Bayesian analyses and bootstrap support values obtained from maximum parsimony and maximum likelihood analyses have been demonstrated and interpreted in different ways (Alfaro et al., 2003; Simmons et al., 2004; Suzuki et al., 2002; Wilcox et al., 2002). We have performed maximum parsimony, including bootstrapping, and Bayesian analyses for all 15 data set combinations and compared the results of these analyses. Since we are employing Bayesian methods to allow model-based phylogenetic inference in a reasonable time, we concentrated on the comparison of bootstrap values under parsimony and Bayesian posterior probabilities in our data set and refrained from performing bootstrap analyses under likelihood.

The goals of the present study include: (1) test the mt LSU as an additional marker for higher-level phylogeny of euascomycetes, (2) evaluate the combination of data sets and the influence on confidence of major clades, and (3) compare the results of the different higher-level phylogenetic studies in inoperculate euascomycetes in maximum parsimony (MP) and Bayesian frameworks. We want to estimate the uncertainty in our knowledge of ascomycete phylogeny and the evolution of morphological characters and the consequences this has for the classification of these organisms.

2. Materials and methods

2.1. Taxon sampling

Data matrices of 53 species of inoperculate euascomycetes and two Saccharomycotina were assembled using sequences of nuclear and mitochondrial small and large subunit rDNA sequences. Ascomycete specimens and sequences used for the molecular analyses are compiled in Table 1. Taxa of seven of the nine classes of inoperculate euascomycetes accepted by Eriksson et al. (2004) were included in this study. We have not been able to include representatives of the classes Laboulbeniomycetes and Orbiliomycetes. Taxon sampling was done to ensure that at least five species per class were included and that each class was represented by species belonging to different groups. We used two species of the Saccharomycotina as out-group since they have been shown to be basal to euascomycetes in numerous studies (e.g., Berbee, 1996; Berbee and Taylor, 1993; Lutzoni et al., 2001, 2004).

2.2. Molecular methods

Total DNA was extracted from freshly collected material, herbarium specimens, or cultures using the DNeasy Plant Mini Kit (Qiagen) following the instructions of the manufacturer. Dilutions (10^{-1} up to 10^{-3}) or undiluted DNA was used for PCR amplifications of the genes coding for the nuclear SSU and LSU rRNA, and the mitochondrial SSU and LSU rRNA, respectively. Primers (nu rDNA primer nomenclature follows Gargas and DePriest, 1996) for amplification were: (a) for the nuclear SSU rDNA: nu-SSU-0021-5′ (Gargas and DePriest, 1996), nu-SSU-0819-5′, nu-SSU-1293-3′, nu-SSU-1750-3′ (Gargas and Taylor, 1992), (b) for the nuclear LSU rDNA: nu-LSU-0155-5′ (Döring et al., 2000), nu-LSU-0042-5′ (= LR0R), nu-LSU-1432-3′ (= LR7), and nu-LSU-1125-3′ (= LR6) (Vilgalys and Hester, 1990), (c) for the mitochondrial SSU rDNA: mr SSU1 (Zoller et al., 1999) and MSU 7 (Zhou et al., 2001), and (d) for the mitochondrial LSU rDNA: ML3.A, ML3.B, ML3.C, ML4, and ML4.A (Printzen, 2002). The 25 μL PCRs contained 2.5 μL buffer, 2.5 μL dNTP mix, 1 μL of each primer (10 μM), 5 μL BSA, 2 μL *Taq*, 2 μL genomic DNA extract, and 9 μL distilled water. Thermal cycling parameters were: initial denaturation for 3 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 52 °C (mt SSU primers) or 53 °C (nu-LSU-0155-5′/LR6), 1 min at 73 °C, and a final elongation for 7 min at 73 °C. Amplification products were viewed on 1% agarose gels stained with ethidium bromide and subsequently purified using the QIAquick PCR Purification Kit (Qiagen) or Nucleo Spin DNA purification kit (Macherey-Nagel).

Fragments were sequenced using the Big Dye Terminator reaction kit (ABI PRISM, Applied Biosystems). Sequencing and PCR amplifications were performed using the same sets of primers. Cycle sequencing was executed with the following program: 25 cycles of 95 °C for 30 s, 48 °C for 15 s, and 60 °C for 4 min. Sequenced products were precipitated with 10 μL of sterile dH_2O, 2 μL of 3 M NaOAc, and 50 μL of 95% EtOH before they were loaded on an ABI 3100 (Applied Biosystems) automatic sequencer. Sequence fragments obtained were assembled with SeqMan 4.03 (DNASTAR) and manually adjusted.

2.3. Sequence alignments

The two mitochondrial data sets contain sequence portions that are highly variable. Standard multiple alignment programs, such as Clustal (Thompson et al., 1994) become less reliable when sequences show a high degree of divergence. Therefore we employed an alignment procedure that uses a linear hidden Markov model (HMM) as implemented in the software sequence alignment and modeling system (SAM) (Karplus et al., 1998) for separate alignments of the four data sets.

Table 1
Species and specimens used in the current study, with Voucher Specimen, Culture, or DNA source information and GenBank Accession Nos.

	Collection/strain	nu SSU	nu LSU	mt SSU	mt LSU
Arthoniomycetes					
Arthoniaceae					
Arthonia dispersa	UPSC 2583	**AY571379**	**AY571381**	**AY571383**	**AY779287**
Roccellaceae					
Combea mollusca	Tehler 7725 (S)	AF043913 +**AY571380**	**AY571382**	**AY571384**	—
Dendrographa minor	USA, California, *Printzen* (hb. Printzen)	AF279381	AF279382	**AY571385**	—
Roccella canariensis	Canary Islands, *Dürhammer E-1741* (hb. Dürhammer)	AF043921	**AY779328**	—	AY048999
Roccella tuberculata	Canary Islands, *Dürhammer E-1736* (hb. Dürhammer)	AF110349	**AY779329**	—	**AY779313**
Schismatomma pericleum	Tehler 7701 (S)	AF138846	AF279408	**AY571390**	**AY779314**
Chaetothyriomycetes					
Chaetothyriaceae					
Ceramothyrium carniolicum	CBS 175.95	AF346418	AY004339	AF346423	**AY779294**
Herpotrichiellaceae					
Berlesiella nigerrima	CBS 513.69	**AY541478**	**AY350579**	**AY35057**	**AY779289**
Capronia mansonii	CBS 101.67	X79318	AY004338	F346422	**AY779292**
Verrucariaceae					
Agonimia tristicula	Slovakia, *Palice 5651* (hb. Palice)	—	AY300828	AY300876	**AY779286**
Norrlinia peltigericola	Ecuador, *Palice 4369* (hb. Palice)	**AY779280**	AY300845	AY300896	**AY779307**
Chaetothyriomycetes incertae sedis					
Glyphium elatum	CBS 268.34	AF346419	AF346420	AF346425	**AY779300**
Dothideomycetes					
Arthopyreniaceae					
Arthopyrenia salicis	CBS 368.94	**AY538333**	**AY538339**	**AY538345**	**AY779288**
Capnodiaceae					
Capnodium citri	CBS 451.66	AY016340	AY004337	F346421	**AY779291**
Dothideaceae					
Dothidea ribesia	CBS 195.58	AY016343	AY016360	**AY538346**	**AY779297**
Stylodothis puccinioides	CBS 193.58	AY016353	AY004342	AF346428	**AY779318**
Hysteriaceae					
Farlowiella carmichaeliana	CBS 206.36	**AY541482**	**AY541492**	**AY571387**	**AY779299**
Hysteropatella clavispora	CBS 247.34	AF164359 +**AY541483**	**AY541493**	**AY571388**	**AY779303**
Myriangiaceae					
Myrangium duriaei	CBS 260.36	AY016347	AY016365	**AY571389**	**AY779305**
Phaeosphaeriaceae					
Phaeosphaeria heterospora	CBS 644.86	AY016354	AY016369	AF346429	**AY779320**
Phaeotrichaceae					
Phaeotrichum benjaminii	CBS 541.72	AY016348	AY004340	**AY538349**	**AY779311**
Pseudoperisporiaceae					
Raciborskiomyces longisetosum	CBS 180.53	AY016351	AY016367	**AY571386**	**AY779312**
Sporormiaceae					
Westerdykella cylindrica	CBS 454.72	AY016355	AY004343	AF346430	**AY779322**
Eurotiomycetes					
Trichocomaceae					
Aspergillus flavus	—	D63696	AF109342	AFU29214	—
Aspergillus nidulans	—	X78539	AF109337	V00653	X0696
Eupenicillium javanicum	—	U21298	AF263348	L14501	—
Eurotium rubrum	CBS 530.65	U00970	AY004346	AF346424	**AY779298**
Penicillium chrysogenum	—	M55628	AF034857	Z23072	D13859
Lecanoromycetes					
Agyriaceae					
Placopsis gelida	—	AF119502	AY212836	AY212859	—
Trapelia placodioides	—	AF119500	AF274103	AF431962	—
Trapeliopsis granulosa	Sweden, *Niemann* (ESS 8684)	AY004349	AF274119	AF381567	**AY779319**
Xylographa vitiligo	Turkey, *Palice* (ESS 21522)	**AY779284**	AY212849	AY212878	**AY779323**
Gyalectaceae					
Gyalecta jenensis	Germany, *Lumbsch & Schmitt* (ESS 21192)	AF279390	AF279391	AF431956	**AY779301**

(*continued on next page*)

Table 1 (*continued*)

	Collection/strain	nu SSU	nu LSU	mt SSU	mt LSU
Lobariaceae					
Lobaria pulmonaria	Canary Islands, *Feige* (ESS-11368)	AF183935	AF183934	AF069541	**AY779304**
Pertusariaceae					
Ochrolechia balcanica	Greece, *Schmitt* (ESS-20968)	**AY779281**	AF329171	AF329170	**AY779308**
Ochrolechia tartarea	Scotland, *Coppins* (ESS 21493)	—	AY 300848	AY300899	**AY779309**
Pertusaria pertusa	Germany, *Killmann*, (ESS-20870)	**AY779282**	AF279300	AF381565	**AY779310**
Ramalinaceae					
Speerschneidera euploca	USA, *Egan 14906* (F)	**AY779283**	AY 300862	AY 300912	**AY779317**
Thelotremataceae					
Diploschistes thunbergianus	Australia, *Eldridge 3800* (F)	AF274112	AF274095	AF431955	**AY779296**
Leotiomycetes					
Cudoniaceae					
Spathularia flavida	CBS 399.52	Z30239	**AY541496**	**AY575101**	**AY779316**
Myxotrichaceae					
Myxotrichum deflexum	CBS 219.50	**AY541480 +AY541481**	**AY541491**	**AY575096**	**AY779306**
Rhytismataceae					
Lophodermium pinastri	—	AF106014	AY004334	AF431957	—
Tryblidiopsis pinastri	CBS 445.71	AF10601	AY004335	AF431963	**AY779321**
Sclerotiniaceae					
Sclerotinia sclerotiorum	CBS 499.50	L37541	AF431951	AF431961	**AY779315**
Sordariomycetes					
Amphisphaeriaceae					
Cainia graminis	CBS 136.62	AF431948	AF431949	AF431952	**AY779290**
Cephalothecaceae					
Cephalotheca sulfurea	CBS 135.34	AF096173	AF431950	AF431953	**AY779293**
Clavicipitaceae					
Beauveria bassiana	—	AF280633	AF391119	U91338	S55628
Diaporthaceae					
Diaporthe phaseolorum	FAU 458	**AY779278**	AY346279	**AY779326**	**AY779295**
Hypocreaceae					
Acremonium sp.	USA, *Huhndorf* (SMH 2748)	**AY779277**	**AY779327**	**AY779325**	**AY779285**
Hypocrea citrina	BEO 9929	**AY779279**	AF399220	**AY779324**	**AY779302**
Lasiosphaeriaceae					
Podospora anserina	—	X54864	—	X14734	X55026
Sordariaceae					
Neurospora crassa	—	X04971	AF286411	Z34001	AF397513
Xylariaceae					
Xylaria hypoxylon	—	U20378	AF132333	AF431964	—
Outgroup					
Saccharomycotina					
Candida albicans	—	X53497	L28817	AF285261	AF285261
Saccharomyces cerevisiae	—	J01353	J01355	X07799	V00699

Newly obtained sequences in bold.

Regions that were not aligned with statistical confidence were excluded from the phylogenetic analysis. In the combined data sets missing sequence portions were coded as "?".

2.4. Phylogenetic analysis

The alignments were analyzed by MP and a Bayesian approach (B/MCMC) (Huelsenbeck et al., 2001; Larget and Simon, 1999).

Maximum parsimony analyses were performed using the program PAUP* (Swofford, 2003). A heuristic search with 200 random taxon addition replicates was conducted with TBR branch swapping and MulTrees option in effect, equally weighted characters, and gaps treated as missing data. Bootstrapping (Felsenstein, 1985) was performed based on 2000 replicates with random sequence additions. To assess homoplasy levels, consistency index (CI), retention index (RI), and rescaled consistency (RC) index (Farris, 1989) were calculated from each parsimony search.

The Bayesian analyses were conducted using the MrBayes 3.0 program (Huelsenbeck and Ronquist, 2001). Posterior probabilities were approximated by sampling trees using a Markov chain–Monte Carlo (MCMC) method. The posterior probabilities of each

branch were calculated by counting its occurrence in trees that were visited during the course of the MCMC analysis.

For all data sets the general time reversible model of nucleotide substitution (Rodriguez et al., 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR + I + G) was used and parameters were calculated for each partition separately as proposed by Nylander et al. (2004). MrBayes was run on each data set producing 2,000,000 generations. Twelve chains were run simultaneously. Trees were sampled every 100 generations for a total of 20,000 trees. The first 100,000 generations (i.e., the first 1000 trees) were deleted as the "burn in" of the chain. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id = tracer) to ensure that stationarity was achieved after the first 100,000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001). Of the remaining 19,000 trees a majority-rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Unlike nonparametric bootstrap values (Felsenstein, 1985), these are estimated probabilities of the clades under the assumed model (Rannala and Yang, 1996) and hence posterior probabilities equal to and above 95% are considered indicative of significant supports. Phylogenetic trees were visualized using the program Treeview (Page, 1996).

We used a Bayesian approach to examine the heterogeneity in phylogenetic signal among the data partitions (Buckley et al., 2002). For the separate genes and the concatenated analyses, the set of topologies reaching 0.95 posterior probabilities were estimated. The combined analysis topology was then examined for conflict with the 0.95 posterior intervals of the single gene analyses. If no conflict was evident, it was assumed that the two data sets were congruent and could be combined. If conflict was evident, the two data sets were interpreted as incongruent, and the concatenated analysis was treated as potentially misleading (Bull et al., 1993).

Three hypothesized phylogenetic relationships of inoperculate euascomycetes expressed in recent studies that were not present in our analyses were tested as null hypotheses using a MCMC tree sampling procedure as described above. The tested alternative topologies were: (1) Arthoniomycetes, Dothideomycetes, and Sordariomycetes forming a monophyletic group (as in Lutzoni et al., 2001, 2004), (2) Dothideomycetes and Chaetothyriomycetes as sister-groups (as in Liu and Hall, 2004), and (3) Chaetothyriomycetes, Eurotiomycetes, and Lecanoromycetes forming a clade (as in Lumbsch et al., 2002; Lutzoni et al., 2001, 2004). For this hypothesis testing, an additional run of the four-region data set with settings as described above was performed to ensure that hypothesis testing and tree sampling for phylogenetic analysis are independent. Two thousand trees at the equilibrium state per null hypothesis were used from this analysis. The probability of the null hypothesis being correct is calculated by counting the presence of this topology in the MCMC sample (Lewis, 2001; Lumbsch et al., 2004). The frequency of trees in the MCMC sample agreeing with the null hypothesis was calculated using the filter command in PAUP* with constraints used to describe the null hypothesis. The constraints were constructed so that only the single node of interest was resolved.

3. Results

Eighty-three new sequences were obtained for this study, including 17 nu SSU, 10 nu LSU, 17 mt SSU, and 39 mt LSU rDNA sequences. These were aligned with sequences obtained from GenBank as shown in Table 1. Summary sequence and tree statistics for individual and combined gene partitions of the maximum parsimony and Bayesian analyses from sequences of 55 taxa are given in Table 2. Ambiguously aligned regions and major insertions, representing spliceosomal and group I introns in the nuclear ribosomal DNA (Bhattacharya et al., 2000; Cubero et al., 2000; Gargas et al., 1995), were excluded from all analyses. Although the length of the unambiguously aligned nucleotide position characters of the four data sets differs considerably between the data sets, the number of variable sites is similar, ranging only from 488 to 569. No significantly supported conflicts were observed between the four data partitions through comparison of the 95% majority-rule consensus trees of the 15 different analyses. This is consistent with the hypothesis that the data partitions have evolved along the same underlying topology. The combined alignment of all four gene partitions is available in Treebase (http://www.treebase.org/treebase).

The maximum parsimony data and indices are given in Table 2. The amount of homoplasy differs between the gene partitions: the nu SSU rDNA exhibits the lowest amount of homoplasy, while the highest amount of homoplasy is present in the mt LSU rDNA gene partition. The higher the number of gene partitions included in the analysis the lower was the number of equally parsimonious trees found. The number of nodes that receive bootstrap support above 74% increases with the number of gene partitions, being eight to 21 in the single gene analyses, 21–27 in the two gene analyses, 29–33 in the three gene analyses, and 36 in the four gene analyses.

The likelihood parameters in the 15 B/MCMC samples (mean values overall gene partitions in combined analyses) are given in Table 2. The base composition varies considerably between the data sets. The nu LSU rDNA was the most GC rich (56.2%). The two mito-

Table 2
Comparison of performance of data partitions under parsimony and in a Bayesian framework

	1-Region				2-Regions						3-Regions				4-Regions
nu SSU	+				+	+	+				+	+	+		+
nu LSU		+			+			+	+		+	+		+	+
mt SSU			+			+		+		+	+		+	+	+
mt LSU				+			+		+	+		+	+	+	+
Aligned length	1642	983	655	601	2625	2297	2243	1638	1584	1256	3280	3226	2898	2239	3881
No. of variable sites	569	488	506	500	1057	1075	1069	994	986	1006	1563	1557	1575	1494	2063
No. MP trees	40	1	5	3	4	1	2	2	5	36	1	12	1	1	1
No of parsimony informative sites	384	395	378	421	779	762	805	773	816	799	1157	1200	1183	1194	1578
No. steps	1624	2593	2629	3064	4219	4282	4594	5173	5628	5780	6799	7261	7451	8328	9950
CI	0.42	0.30	0.30	0.30	0.34	0.34	0.30	0.30	0.30	0.30	0.33	0.32	0.32	0.30	0.32
RI	0.62	0.57	0.50	0.40	0.58	0.54	0.47	0.52	0.48	0.44	0.54	0.47	0.46	0.48	0.50
RC	0.31	0.19	0.17	0.13	0.22	0.20	0.15	0.18	0.15	0.14	0.20	0.17	0.17	0.16	0.18
No. nodes with bootstrap $\geqslant$ 75%	18	19	21	8	25	26	24	27	24	21	33	29	30	31	36
Mean likelihood	−10923	−13101	−12055	−12713	−24319	−23637	−23639	−25288	−26343	−25159	−36657	−37842	−38139	−38824	−50449
$\pi A_{mean/all}$	0.25	0.23	0.357	0.447	0.243	0.289	0.336	0.28	0.326	0.408	0.266	0.279	0.301	0.326	0.292
$\pi C_{mean/all}$	0.213	0.238	0.13	0.100	0.277	0.181	0.155	0.174	0.157	0.106	0.193	0.189	0.167	0.15	0.175
$\pi G_{mean/all}$	0.275	0.324	0.193	0.126	0.29	0.244	0.238	0.264	0.241	0.151	0.267	0.237	0.244	0.232	0.247
$\pi T_{mean/all}$	0.262	0.208	0.321	0.327	0.241	0.286	0.271	0.288	0.277	0.335	0.274	0.275	0.288	0.293	0.285
$r(AC)_{mean/all}$	1.397	0.837	1.025	0.785	1.048	1.123	1.014	1.153	1.004	1.039	1.153	1.128	1.133	1.116	1.142
$r(AG)_{mean/all}$	2.621	2.298	2.781	1.821	2.483	2.823	2.513	2.945	2.303	2.385	2.816	2.563	2.528	2.587	2.695
$r(AT)_{mean/all}$	1.263	1.401	1.476	1.139	1.221	1.816	1.923	1.977	2.591	1.311	1.793	2.599	2.432	2.346	2.398
$r(CG)_{mean/all}$	0.806	0.954	0.652	0.815	1.003	0.672	0.923	1.262	1.446	0.863	1.018	1.074	1.091	1.24	1.011
$r(CT)_{mean/all}$	5.396	6.517	4.268	2.319	5.348	4.283	4.824	5.731	5.278	3.522	5.048	4.668	4.873	4.969	4.581
$\alpha_{mean/all}$	0.592	0.69	0.573	0.547	0.575	0.522	0.599	0.619	0.709	0.577	0.566	0.572	0.591	0.684	0.573
$P(invar)_{mean/all}$	0.398	0.371	0.042	0.035	0.389	0.309	0.292	0.216	0.268	0.064	0.332	0.35	0.294	0.206	0.294
No. of nodes with pp $\geqslant$ 0.95	19	29	30	16	37	36	34	35	35	31	40	38	38	38	41
Arthoniomycetes[a]	–/–	99/1.0	83/1.0	–/0.96	100/1.0	99/0.99	100/1.0	100/1.0	100/1.0	75/0.97	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0
Chaetothriomycetes	99/1.0	94/0.99	99/1.0	–/–	100/1.0	100/1.0	98/1.0	100/1.0	–/1.0	92/1.0	100/1.0	100/1.0	100/1.0	96/1.0	100/1.0
Dothideomycetes	–/–	–/–	–/–	–/–	–/–	–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	– (58)/0.95
Eurotiomycetes	100/1.0	100/1.0	99/1.0	–/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0
Lecanoromycetes	–/–	–/1.0	–/0.99	–/–	76/1.0	58/0.96	75/0.95	–/–	–/0.99	– /1.0	84/1.0	77/0.99	81/1.0	–/1.0	82/1.0
Leotiomycetes	–/–	–/–	–/–	–/0.96	–/–	72/1.0	76/–	86/–	93/0.99	76/0.96	83/0.96	92/0.99	94/0.99	96/1.0	98/1.0
Sordariomycetes	100/1.0	99/1.0	83/1.0	75/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	88/0.98	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0
Clade I (Leo+S)	–/–	–/1.0	–/0.95	–/–	–/0.95	–	–/–	75/1.0	–/1.0	–/–	–/1.0	–/1.0	89/1.0	–/1.0	75/1.0
Clade II (A+D)	–/–	–/0.99	–/–	–/–	–/1.0	–	–/–	–/–	–/0.99	–/–	–/1.0	–/0.99	–/1.0	–/–	76/1.0
Clade III (C+E)	–/0.98	–/1.0	76/1.0	–/–	81/1.0	93/1.0	91/0.99	96/1.0	–/1.0	76/0.98	93/1.0	79/1.0	89/1.0	76/1.0	85/1.0
Leotiomyceta	100/1.0	100/1.0	100/1.0	97/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0	100/1.0

[a] Maximum parsimony bootstrap support above 74% and Bayesian posterior probability support above 94% indicated, – means no strong support.

chondrial data sets had a much lower GC content, being 22.6% in the mt LSU and 32.3% in the mt SSU rDNA. We tested for inequalities of nucleotide composition within the data sets (data not shown), but found no significantly deviating sequences using a χ^2 test implemented in Tree–Puzzle (Strimmer and von Haeseler, 1996). The percentage of invariable sites also differs between the data sets, being highest in the nu SSU rDNA (39.8%), while only 3.5% of the positions in the mt LSU rDNA are invariable. The gamma shape parameter α is similar in the gene partitions, with the exception of the nu LSU rDNA that shows a higher value (0.69) than the other three partitions. The number of nodes that receive posterior probability support above 94% increases with the number of gene partitions combined, being 16–30 in the single gene analyses, 31–37 in the two gene analyses, 38–40 in the three gene analyses, and 41 in the four gene analyses.

Parsimony analysis of the four-region data set yielded one most parsimonious tree (9950 steps, CI = 0.32, RI = 0.50, RC = 0.18, Table 2). The topology was almost identical to the 50% majority-rule consensus tree obtained from the B/MCMC tree sampling in a Bayesian framework, which is shown in Fig. 1. There were no differences in the monophyly and relationships of the classes. The few differences included only relationships within classes that were not strongly supported in either analysis. The mean likelihood of the trees in the sampling was ln = −50,449 (Table 2). The seven classes recognized by Eriksson and Winka (1997) were all resolved as monophyletic, with bootstrap support (BP) ranging from 58% (Dothideomycetes) to 100% (Arthoniomycetes, Chaetothyriomycetes, Eurotiomycetes, and Sordariomycetes) and posterior probabilities (pp) of 1.0 for all classes except Dothideomycetes that received a pp of 0.95 (Fig. 1, Table 2). The higher-level relationships among the seven classes were only partially resolved with confidence. The following classes are strongly supported as sister-groups under parsimony and in a Bayesian framework: Arthoniomycetes and Dothideomycetes (BP 76%, pp 1.0), Chaetothyriomycetes and Eurotiomycetes (BP 85%, pp 1.0), and Leotiomycetes and Sordariomycetes (BP 75%, pp 1.0). The Lecanoromycetes are basal to a group that includes the Arthoniomycetes/Dothideomycetes and the Leotiomycetes/Sordariomycetes clades. However, neither the sister-group relationship of these two two-class clades nor the position of the Lecanoromycetes is strongly supported. To ensure that this lack of support is not due to missing data in one gene partition of some taxa, we performed additional four-region analyses employing both MP and B/MCMC with the core data set only (data not shown). The tree topology and the strong support values for the major nodes were identical in these additional analyses.

The inoperculate euascomycetes (= Leotiomyceta) are strongly supported as monophyletic in both analyses (BP 100%, pp 1.0). Support for the internal topologies of the classes is mostly robust with only a few exceptions. However, given the poor taxon sampling for a study of relationships within the classes (only 5–11 species per class were included in this study), we refrain from discussing the internal topologies any further.

Since the topology revealed in the combined analysis of the four gene partitions is not congruent with relationships proposed in some recent publications, the power of the combined data set to reject these alternative topologies was tested in a Bayesian framework. Topologies with (1) Arthoniomycetes, Dothideomycetes, and Sordariomycetes and (2) Dothideomycetes and Chaetothyriomycetes (= Loculoascomycetes) forming each a monophyletic group are rejected at $p < 0.0001$*, while a monophyly of Chaetothyriomycetes, Eurotiomycetes, and Lecanoromycetes cannot be rejected ($p = 0.38$).

Phylogenetic relationships of the 53 species in the Leotiomyceta were estimated using the four-region data set and all 14 possible subpartitions of the data (Table 2). Based on the number of nodes supported above 74% bootstrap and above 94% posterior probability support, the most decisive data partition was the mt SSU rDNA (21/30 strongly supported nodes), while the mt LSU rDNA showed the lowest amount of strongly supported nodes (8/16). The most robust two-region data partitions were the combination of nu SSU and nu LSU rDNA in a Bayesian framework (37 nodes with pp above 94%) and a combination of nu LSU and mt SSU rDNA in a maximum parsimony framework (27 nodes above 74% bootstrap support); the most robust three-region data partition was the nu SSU/nu LSU/mt SSU rDNA data partition under parsimony and in a Bayesian framework (33/40 strongly supported nodes). The four-region data set was the most decisive overall: 36 nodes received bootstrap support above 74% and 41 nodes above 94% posterior probability support. As stated above both the bootstrap and posterior probability support values increase with the number of gene partitions included in the analyses. However, in all analyses the number of nodes with significant posterior probability support are higher than those with strong bootstrap values; the difference varies between 12 nodes in the two-region analysis of a combination of nu SSU and nu LSU rDNA and only one node in the nu SSU rDNA single gene analysis. In the combined analyses, the differences in the number of strongly supported nodes between bootstrap and posterior probabilities tends to decrease: in the two-region analyses eight to 12 nodes less receive strong bootstrap support, in the three-region analyses seven to nine nodes less receive strong bootstrap support, while in the four-region analysis the difference is only five nodes.

The monophyly of Leotiomyceta is strongly supported in all 30 analyses under parsimony and in a Bayesian framework (BP 97–100%, pp 1.0). Other relationships between different classes varied considerably

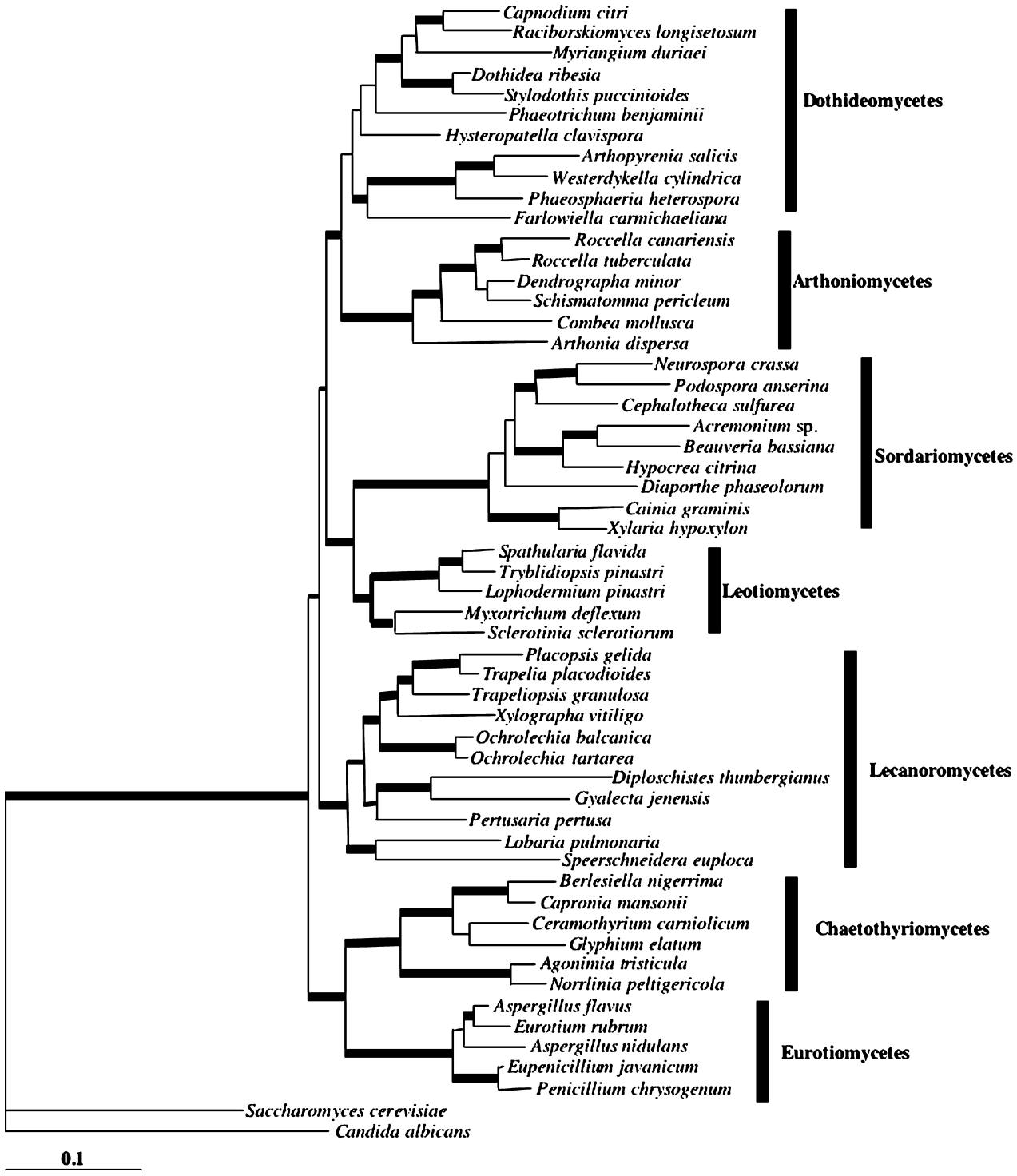

Fig. 1. Phylogeny of inoperculate euascomycetes as inferred from a four gene-partition analysis. This is a 50% majority-rule consensus tree based on 19,000 trees from a B/MCMC tree sampling procedure. Branches with posterior probabilities equal or above 0.95 and MP bootstrap support values above 74% are indicated in bold. Classes as accepted by Eriksson et al. (2004) indicated at margin.

between the groups and the parsimony and Bayesian frameworks (Table 2). A sister-group relationship of Chaetothyriomycetes and Eurotiomycetes is strongly supported in all Bayesian analyses except the analysis of the mt LSU rDNA alone, bootstrap support above 74% is lacking in three of the one-region analyses, and the combined data set of nu LSU and mt LSU rDNA. A sister-group relationship between Leotiomycetes and Sordariomycetes is strongly supported in the four-gene analysis under parsimony and in a Bayesian framework, but only in a Bayesian framework in two of the four single gene analysis, and in two under parsimony and seven

in a Bayesian framework of the two- and three-gene analyses. A sister-group relationship of Arthoniomycetes and Dothideomycetes is only strongly supported in eight of the 30 separate analyses and only in one of the single gene studies. Under parsimony only the four-region analysis strongly supports this relationship. Similar variation of support was found for the classes recognized by Eriksson and Winka (1997). While the Dothideomycetes are strongly supported in the four-region analyses in a Bayesian framework, they lack strong support under parsimony (BP 58% in the four-region analysis of the combined data set). In contrast, the Sordariomycetes are strongly supported in all analyses performed.

4. Discussion

The purposes of this study was to evaluate the utility of mt LSU rDNA for the elucidation of the higher-level phylogeny of inoperculate euascomycetes, analyze the combination of four ribosomal gene partitions, evaluate the differences of MP-bootstrapping and Bayesian posterior probabilities, and compare different recent higher-level studies to evaluate our current knowledge of euascomycete evolution.

The mt LSU gene partition was outperformed by all other single gene partition analyses under parsimony and in a Bayesian framework in terms of number of supported clades and homoplasy. However, when added to the other two- or three-partitions data sets the mt LSU increased the number of supported clades, indicating that this gene partition has a resolving power for the elucidation of higher-level phylogeny in euascomycetes when used in combined analyses. There is no indication that mt LSU sequence data should only be used in studies at lower taxonomic levels in ascomycetes. Similar results were shown by Binder and Hibbett (2002) in a study of homobasidiomycetes in which a mt LSU single gene data set also performed poorly in comparison with other ribosomal gene partitions, but enhanced the number of supported clades in most concatenated analyses.

The nuclear and mitochondrial ribosomal gene partitions supported the same overall topology and no intra- or intergenomic conflict was found. The four-region data set provided the most robust support of the inoperculate euascomycete phylogeny overall. Further, the results of our study indicate that combined data sets are necessary to resolve higher-level phylogenetic relationships in ascomycetes.

Differences in bootstrap and Bayesian support values were demonstrated using simulated (Alfaro et al., 2003; Cummings et al., 2003; Douady et al., 2003; Suzuki et al., 2002; Wilcox et al., 2002) and empirical (Douady et al., 2003; Simmons et al., 2004) data. The interpretation of these differences differed between the authors, favoring either Bayesian support values (Alfaro et al., 2003; Wilcox et al., 2002), or bootstrap or jackknife values (Cummings et al., 2003; Simmons et al., 2004; Suzuki et al., 2002), culminating in the statement that Bayesian support values should not be interpreted as probabilities that clades are correctly resolved (Simmons et al., 2004). Douady et al. (2003) regarded the bootstrap values as a reliable lower bound and Bayesian support values as an upper bound. Our results also show differences in bootstrap values and posterior probabilities and confirm that the latter are always higher than bootstrap values. However, we cannot find fundamental differences in the behavior of the two methods in estimating confidence of nodes in our study. While bootstrap support values are generally lower, they appear to converge to some extent with the number of gene partitions analyzed. In this context we only consider bootstrap support above 74% following Hillis and Bull (1993) and posterior probabilities above 94% following Rannala and Yang (1996). Values above these margins are regarded as indicative of strong support. If the presence of these strong support values is compared and not the actual values (which are naturally different since bootstrapping is not a statistical method), it appears that the behavior of the two methods is comparable in our study. However, bootstrapping was more conservative and required a greater number of characters to converge to a similar number of strongly supported nodes. This is readily explained by the fact that bootstrapping includes partial rearrangements of the data and therefore inherently measures presence of phylogenetic signal in multiple shorter subsets of the original data set. Thus, while we find bootstrap values to be underestimates, we view them as helpful lower bounds of support values, in agreement with Douady et al. (2003). In our study posterior probabilities do not appear to be overestimates, since most supported nodes also receive strong bootstrap support under parsimony in the four-region analysis. However, Bayesian analyses appear more efficient in detecting phylogenetic signals than MP-bootstrap by requiring fewer nucleotides to obtain strongly supported nodes. This is evident in the differences in support for clades I and II in the two- and three-partitions analyses. They are strongly supported in seven, resp. five analyses in a Bayesian framework, but only in two or none under parsimony. However, these two clades are strongly supported in both four-partition analyses. We also find it advantageous that posterior probability values are straightforward statistical values that are easy to interpret.

The four-partition analyses strongly supported the monophyly of the classes proposed by Eriksson and Winka (1997) with the exception of the Dothideomycetes that is poorly supported under parsimony. This indicates that further studies are necessary to resolve the phylogeny of this group of fungi and to test its monophyly.

A comparison of results obtained in five recent higher-level phylogenetic studies (including this study) in euascomycetes (Table 3) shows a large amount of uncertainty above the level of classes as recognized by Eriksson and Winka (1997). None of the five studies agree with one another in all supported relationships. The highest similarities between the studies do not correspond to the genes examined, but apparently to whether they are performed by the same researchers. This suggests that taxon sampling is a major issue in finding explanations for the deviating results. Given this large amount of uncertainty it appears premature to suggest changes in the classification by Eriksson and Winka (1997), such as the merging of the Chaetothyriomycetes and Eurotiomycetes into Eurotiomycetes s. lat. (Reeb et al., 2004) or the inclusion of Arthoniomycetes and Dothideomycetes as subclasses into Sordariomycetes s. lat. (Lutzoni et al., 2004; Reeb et al., 2004). The latter relationship is only supported in two of five recent studies, while the former is not found in a study employing RPB-2 sequence data (Liu and Hall, 2004).

Given the uncertainty of the higher-level phylogeny in euascomycetes, we can only draw limited conclusions about the morphological evolution in these fungi. As already shown in early studies (e.g., Gargas and Taylor, 1995; Gargas et al., 1995a; Lutzoni et al., 2001), apotheciate taxa form a paraphyletic group and lichen-forming (mostly classified in Lecanoromycetes) and non-lichenized discomycetes (mostly classified in Leotiomycetes and Pezizomycetes) are not closely related. Non-lichenized taxa with perithecia appear to form a monophyletic group and seem to be derived. These species are classified in the Sordariomycetes. Species with cleistothecia appear in different groups (e.g., Geiser and LoBuglio, 2001; Saenz et al., 1994; Suh and Blackwell, 1999), but a core group of these fungi form a monophyletic class Eurotiomycetes. A major challenge are the ascoloculoarous fungi. Fungi that start their ascoma development with the development of a stroma prior to the dikaryotization were distinguished as ascolocularous by Nannfeldt (1932) and later classified into a class Loculoascomycetes by Luttrell (1955). Loculoascomycetes were found to belong to two unrelated classes according to ribosomal sequence studies (e.g., Berbee, 1996; Lindemuth et al., 2001; Winka, 2000; Winka et al., 1998): Chaetothyriomycetes and Dothideomycetes. In a study employing RPB-2 sequences, Liu and Hall (2004) found the loculoascomycetes forming a monophyletic clade. However, their interpretation that all species studied by them and included in this monophyletic clade have an ascolocularous development is an oversimplification. The lichen-forming Arthoniomycetes, which clustered with the Dothideomycetes, have been shown to have an ascohymenial ascoma development (e.g., Henssen and Thor, 1994). Further the Verrucariales, which are also mostly lichen-forming and clustered with the Chaetothyriales—in agreement with other molecular studies (e.g., Lumbsch et al., 2004; Lutzoni et al., 2004)—are also known to have an ascohymenial ascoma development (e.g., Doppelbauer, 1960; Janex-Favre, 1970). This suggests that additional ontogenetic studies in non-lichenized loculoascomycetes are urgently needed to better understand the occurrence of this fundamental character set. Further these uncertainties indicate that our knowledge of morphological diversity in ascomycetes is currently not sufficient to allow a reliable reconstruction of body plan evolution in these fungi. In particular a re-evaluation of the ascoma development in the Chaetothyriales appears to be necessary, since closely related groups, such as Verrucariales and also the Pyrenulales (Lutzoni et al., 2004) exhibit ascohymenial ascoma development (e.g., Janex-Favre, 1970).

Although, a large number of nodes received strong support in the four-region analyses, the backbone of the phylogeny of inoperculate euascomycetes remained unresolved with confidence as in most recent multi-gene

Table 3
Comparison of relationships between classes in four recently published higher-level phylogenetic studies and this study

Supraordinal relationship	Lutzoni et al. (2001) (nu SSU + nu LSU)	Lumbsch et al. (2002) (nu SSU, nu LSU + mt SSU)	Liu and Hall (2004) (RPB-2)	Lutzoni et al. (2004) (nu SSU, nu LSU, mt SSU + RPB-2)	This study (nu SSU, nu LSU, mt SSU + mt LSU)
Number of inoperculate euascomycete taxa included	45	27	41	58	53
Arthoniomycetes + Dothideomycetes	–	(–)	+	(+)[a]	+
Chaetothyriomycetes + Eurotiomycetes	+	+	–	+	+
Chaetothyriomycetes + Dothideomycetes	–	–	+	–	–
Leotiomycetes + Sordariomycetes	–	+	+	–	+
Arthoniomycetes + Dothideomycetes + Sordariomycetes	+	(–)	–	+	–
Chaetothyriomycetes + Eurotiomycetes + Lecanoromycetes	+	(+)	–	+	–

+, Significantly supported in Bayesian analysis; (+) present in 50% majority-rule consensus tree of Bayesian analysis, but lacking significant support; –, not present in 50% majority-rule consensus tree of Bayesian analysis; and (–) one group not included in study.

[a] Significant support in a three-gene analysis in the same paper.

studies. This lack of confidence in the backbone of euascomycete phylogeny led Berbee et al. (2000) to the pessimistic conclusion that no amount of data may resolve the rapid radiation of euascomycetes. However, the results of our study show steady increase in the resolution and support of nodes with increasing amount of data, which give us hope that with the addition of further gene regions—as planned in the current AFTOL project (Lutzoni et al., 2004)—the early radiation of the euascomycetes will finally be uncovered.

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