Contribution of RPB2 to multilocus phylogenetic studies of the euascomycetes (Pezizomycotina, Fungi) with special emphasis on the lichen-forming Acarosporaceae and evolution of polyspory

Valérie Reeb, François Lutzoni, and Claude Roux

Abstract

Despite the recent progress in molecular phylogenetics, many of the deepest relationships among the main lineages of the largest fungal phylum, Ascomycota, remain unresolved. To increase both resolution and support on a large-scale phylogeny of lichenized and non-lichenized ascomycetes, we combined the protein coding-gene RPB2 with the traditionally used nuclear ribosomal genes SSU and LSU. Our analyses resulted in the naming of the new subclasses Acarosporomycetidae and Ostropomycetidae, and the new class Lichinomycetes, as well as the establishment of the phylogenetic placement and novel circumscription of the lichen-forming fungi family Acarosporaceae. The delimitation of this family has been problematic over the past century, because its main diagnostic feature, true polyspory (numerous spores issued from multiple post-meiosis mitoses) with over 100 spores per ascus, is probably not restricted to the Acarosporaceae. This observation was confirmed by our reconstruction of the origin and evolution of this form of true polyspory using maximum likelihood as the optimality criterion. The various phylogenetic analyses carried out on our data sets allowed us to conclude that: (1) the inclusion of phylogenetic signal from ambiguously aligned regions into the maximum parsimony analyses proved advantageous in reconstructing phylogeny; however, when more data become available, Bayesian analysis using different models of evolution is likely to be more efficient; (2) neighbor-joining bootstrap proportions seem to be more appropriate in detecting topological conflict between data partitions of large-scale phylogenies than posterior probabilities; and (3) Bayesian bootstrap proportion provides a compromise between posterior probability outcomes (i.e., higher accuracy, but with a higher number of significantly supported wrong internodes) vs. maximum likelihood bootstrap proportion outcomes (i.e., lower accuracy, with a lower number of significantly supported wrong internodes).

1. Introduction

1.1. Addition of RPB2 to resolve the phylogeny of the euascomycetes

The Ascomycota is the largest of the four fungal phyla, including over 32,000 recognized species distributed among 53 orders (Eriksson et al., 2004), with 48 orders part of the subphylum Pezizomycotina (euascomycetes), one order part of the Saccharomycotina (hemiascomycetes), and four orders part of the Taphrinomycotina (archiascomycetes). Forty-two percent of the known species of ascomycetes are lichen-forming fungi (Kirk et al., 2001). Reconstruction of broad phylogenies of the lichenized and non-lichenized ascomycetes, based on single (Lumbsch et al., 2001; Schultz et al., 2001; Stenroos and DePriest, 1998; Tehler et al., 2003) or multiple ribosomal RNA genes (Bhattacharya et al., 2000; Kauff and Lutzoni, 2002; Lucking et al.,
Recent research has clearly demonstrated that the use of protein-coding genes can contribute greatly to resolving deep phylogenetic relationships with high support, and/or increased support for topologies inferred using ribosomal RNA genes in fungal phylogeny (Liu et al., 1999; Matheny et al., 2002; O’Donnell et al., 2001; Tanabe et al., 2004). However, there has been minimal use of protein-coding genes to reconstruct ascomycete phylogenies and the taxon sampling generally has been limited in terms of numbers of specimens sequenced and orders represented (Craven et al., 2001; Geiser et al., 1998; Landvik et al., 2001; Liu et al., 1999; Myllys et al., 2002; Thell et al., 2002; Yun et al., 1999; see introduction by Lutzoni et al., 2004). One exception is found in Liu and Hall (2004), who reconstructed a phylogeny for 54 lichenized and non-lichenized ascomycetes based on the second largest RNA polymerase subunit (RPB2). However, because they restricted their analysis to this protein-coding gene, it was still unknown how much this gene contributes to the resolution and support of relationships within the Ascomycota when combined with other genes such as the nuclear ribosomal small and large subunit RNA genes (SSU nrDNA and LSU nrDNA). Lutzoni et al. (2004) conducted a three-locus-based phylogenetic study (SSU nrDNA, LSU nrDNA, and RPB2) of 157 species and a four-locus-based study (SSU nrDNA, LSU nrDNA, mitochondrial small subunit RNA gene, and RPB2) of 103 species representing a broad spectrum of the taxonomic diversity within the Basidiomycota and Ascomycota. One of the main goals of our study was to evaluate the contribution of RPB2 when combined with SSU and LSU nrDNA and when phylogenetic analyses are restricted to the Ascomycota in resolving and improving phylogenetic confidence for deep relationships within the euascomycetes (Pezizomycotina). To this day, these have been the most problematic relationships to resolve with high confidence within the Ascomycota.

1.2. True polyspory and the circumscription of the Acarosporaceae

Lichen-forming fungi placed in the family Acarosporaceae generally form crustose thalli growing on rocks, are often found in dry habitats and are distributed worldwide. Zahlbruckner (1907) originally created the family based on the assumption that lichenized genera having irregular discs and polyspored asci were probably phylogenetically related. Consequently, the main diagnostic feature upon which taxonomists have relied to distinguish the Acarosporaceae is the number of spores produced per ascus. Typically, ascomycetous fungi produce eight ascospores per ascus (Alexopoulos et al., 1996), resulting from meiosis followed by a mitotic division that provides eight nuclei around which ascospore initials form. In the case of the Acarosporaceae, meiosis is followed by several mitoses (true polyspory), generally producing over 100 single-celled spores per ascus. Over the past, this arbitrary criterion of >100 spores per ascus was adopted by taxonomists to readily identify members of the family Acarosporaceae (Golubkova, 1988; Hafellner, 1995; Ozenda and Clauzade, 1970). A few exceptions exist: for example, Acarospora macrospora, A. oligospora, A. plagiocladiformis, and Glypholecia scabra all have fewer than 100 spores per ascus but are, nevertheless, classified within the Acarosporaceae based on various other morphological characters.

The delimitation of the family based on true polysporory with over 100 spores per ascus (TP > 100), which assumes that TP > 100 originated only once during Ascomycota evolution, has led to a very heterogeneous circumscription of the Acarosporaceae. Morphological differences between members of the family were observed based on thallus development (Golubkova, 1988), apothecium structure (Dodge, 1973; Elenkin, 1911), ascus structure (Hafellner, 1995) or ontogeny of the apothecium (Vezda, 1978), among others (see also Eriksson and Hawksworth, 1996). According to Hafellner (1995) and Ozenda and Clauzade (1970), true polysporory is well known from the lichen order Lecanorales (Lecanoromycetes, Ascomycota) where the Acarosporaceae have been placed. It is therefore possible that true polysporory has evolved several times during the evolution of lichenized ascomycetes, thus explaining this morphological heterogeneity. As a consequence, several authors have used different diagnostic features in attempting to re-circumscribe the family (Golubkova, 1988; Hafellner, 1995; Magnusson, 1936), and over the past century, the number of genera included in the Acarosporaceae has fluctuated from five (Zahlbruckner, 1907) to 14 (Kirk et al., 2001; Table 1).

Only the genera Acarospora and Glypholecia, as well as Sarcogyn (as a genus or subgenus), are constantly present across published classifications of the Acarosporaceae (Table 1). Three other genera (Lithoglypha, Polysporina, and Thelocarpella), described after the establishment of the Acarosporaceae, also have been placed consistently within the family. The placement of other genera with polyspored asci is more problematic, as they are constantly moved in and out of the Acarosporaceae, considered at different taxonomic levels, or split into additional genera, depending on the classifications (Table 1). Moreover, taxa excluded from the Acarosporaceae were not always given a new and stable placement. For example, Biatoridium and Timdalida are
Table 1
Genera included in the Acarosporaceae according to selected classifications and a new classification proposed here

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<td>Acarospora A. Massal. (1852)</td>
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<tr>
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<td>Polyspora Vezda (1878)</td>
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<td>Strangospora Köber (1860)</td>
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<td>Thelocarpella Nav.-Ros. &amp; Cl. Roux (1999)</td>
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<tr>
<td>Thelocarpus Nyl. (1853)</td>
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<td>Timdalia Hafellner (2001)</td>
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When possible, synonymy of taxa was taken into account for each classification. "\(\^a\)" taxa present in a given classification. "\(\^b\)" taxa included in a genus considered part of the Acarosporaceae at the time that the classification was proposed, but that were subsequently transferred to different genera that were sometimes part of a different family. "\(\^c\)" taxa not part of the Acarosporaceae. "\(\^d\)" taxa that did not exist at the time that the classification was proposed, that were part of a different genus outside the Acarosporaceae, or that were not mentioned in a given classification. "\(\^?\)" taxon for which we are unable to determine the systematic position with high confidence. "na", taxa for which we were unable to gather material or data.

\(^a\) = included in Acarospora.
\(^b\) = included in Biatoridium.
\(^c\) = included in Sarcogyne.
\(^d\) = included in Thelocarpus.
both regarded, at the moment, as Lecanorales genera incertae sedis (Eriksson et al., 2004), and the placement of the Thelocarpaceae, where both Thelocarpon and Sarcosagium are sometimes classified (Eriksson et al., 2004; Hafellner, 1995), remains uncertain (Eriksson et al., 2004). Finally, the placement of Endocena, Eschatogonia, and Phacopsis in the Acarosporaceae (Eriksson, 1999; Eriksson et al., 2001; Hawksworth et al., 1995; Kirk et al., 2001) is quite surprising, as polyspored ascii have never been observed for these genera. Recent studies have placed Endocena in the Icamdophiliaceae (Stenroos et al., 2002) and Phacopsis in the Parmeliaceae (Persóh and Rambold, 2002). The placement of Eschatogonia is still uncertain, but it may be related to the Bacidiaceae (Ekman, 1996), or Ramalinaceae (Hertel and Rambold, 1995). A stable circumscription of this cryptic family of lichen-forming fungi is greatly needed at this time.

1.3. Phylogenetic placement of the Acarosporaceae within the Ascomycota

According to most recent classifications, the family Acarosporaceae belongs in the Lecanoromycetidae, order Lecanorales (Kirk et al., 2001), suborder Acarosporineae, together with the Hymeneliaceae (Eriksson et al., 2004; Tehler, 1996) which do not have polyspored ascii. However, these two families have several putatively ancestral features (e.g., a crustose saxicolous thallus, a cryptolecaneanorine apothecium, and a non-amyloid tholus), which suggest a more basal position within the lichenized ascomycetes than previously thought (i.e., Lecanorales). Based on molecular data, Stenroos and DePriest (1998) hypothesized that the genus Acarospora might fall outside the Lecanorales s. str. More recent multi-gene phylogenies (Kauff and Lutzoni, 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004) showed that the Acarosporaceae form a separate lineage outside the Lecanorales, but still fall within the Lecanoromycetes. However, its relationship with other members of the Lecanoromycetes has not been sufficiently supported to draw any strong conclusions as to its placement.

1.4. Objectives of this study

The purpose of the present study was to reconstruct a robust phylogeny for the lichenized and non-lichenized euascomycetes by combining a protein-coding gene with ribosomal RNA genes, with the specific goals of: (1) resolving deep relationships within the Pezizomycotina and revising the classification of supraordinal taxa within that subphylum; (2) circumscribing the family Acarosporaceae and establishing its placement within the Pezizomycotina; (3) establishing the phylogenetic placement of genera with TP > 100 that do not belong to the Acarosporaceae s. l.; and (4) reconstructing the origin and evolution of true polyspory with over 100 spores per ascus.

In order to reconstruct the phylogeny of the euascomycetes and to estimate branch support on the topology recovered, we implemented several types of analyses and compared: (1) the performance of maximum parsimony (MP) when incorporating signal from ambiguously aligned regions with Bayesian inference restricted to unambiguously aligned regions; (2) different methods to detect phylogenetic conflict among the three loci (SSU nrDNA, LSU nrDNA, and RPB2) used in this study as part of a conditional combinability approach (Bull et al., 1993); and (3) the newly proposed Bayesian bootstrap proportion (B-BP; Douady et al., 2003a) to posterior probability (PP) and maximum likelihood bootstrap proportion (ML-BP) as measures of phylogenetic support.

2. Materials and methods

2.1. Taxon sampling

To circumscribe and determine the phylogenetic placement of the family Acarosporaceae s. str. within the Ascomycota, 89 taxa were selected (Supplementary material 1). Twenty-five taxa belong to the family Acarosporaceae sensu auct. (e.g., Eriksson et al., 2004; Eriksson and Hawksworth, 1991; Golubkova, 1988; Hafellner, 1995; Kirk et al., 2001; Magnusson, 1936; Poelt, 1973; Tehler, 1996; Zahlbruckner, 1907), representing 12 of the 21 genera shown in Table 1. Six of the remaining genera, i.e., Ahlesia, Biatorella, Lithoglypha, Maronella, Melaphloea, and Timdalid, could not be included due to a lack of material. The three eight-spored genera listed in Table 1 (Endocena, Eschatogonia, and Phacopsis) were not investigated because their placement in the Acarosporaceae is very unlikely (see Section 1.2). The remaining 63 Ascomycota specimens were selected either to serve as a broad basis for inferring ascomycete phylogeny or to assess hypotheses of relationships among members of the Acarosporaceae sensu auct., as proposed, for example, by Eriksson et al. (2004), Eriksson and Hawksworth (1991), Hafellner (1993, 1995), Hertel and Rambold (1988), and Tehler (1996). Overall, 24 of the 53 orders listed by Eriksson et al. (2004) are represented here, as are four unclassified families (according to Eriksson et al., 2004) of lichenized ascomycetes (Baeomycetaceae, Icamdophiliaceae, Thelocarpaceae, and Umbilicariaceae). One species in the Basidiomycota was used to root the Ascomycota tree.

2.2. Molecular data

Genomic DNA was isolated from well-preserved herbarium specimens and fresh fungal cultures using the
Purgene Kit (GENTRA Systems) following the manufacturer’s protocol for filamentous fungi. Symmetric polymerase chain reactions (PCR) were performed to amplify the targeted genes: a 1.2-kb fragment at the 5’ end of the SSU nrDNA; a 1.4-kb fragment at the 5’ end of the LSU nrDNA; and a 2.1-kb fragment (in two parts, from conserved regions 5 to 7 and 7 to 11, following Liu et al., 1999) of the protein-coding gene RPB2 nDNA. Combinations of various primers were used to amplify the SSU (nssu97a, nssu97b, nssu131, NS22, nssu1088), LSU (LR0R, LIC15R, LIC24R, LIC2044, and LR7), and RPB2 (RPB2-5F, RPB2-7cF, RPB2-11aR, RPB2-608F, RPB2-3053R, and RPB2-3053bR) genes (see Supplementary material 2; Kauff and Lutzoni, 2002; and http://www.lutzonilab.net/pages/primer.shtml).

Amplification reactions were prepared for a 50 μl final volume containing 25 μl PCR Master Mix (Promega #M7505), 2.5 μl for each of the 10 μM SSU/LSU primers or 4 μl for each of the 10 μM RPB2 primers, 1 μl of 10 mg/ml bovin serum albumin (New England Biolabs #B9001S), 1 μl of 25 mg/ml MgCl₂, 17 μl or 14 μl of sterile double-distilled water for the SSU/LSU or RPB2 reactions, respectively, and 1 μl of template genomic DNA. PCR was performed on Peltier Thermal Cyclers PTC-200 (MJ Research) under the following conditions: one cycle of 1 min at 95°C linked to 25 cycles of 45 s at 95°C, 40 s at 52°C, and 2 min at 72°C, followed by 15 cycles of 45 s at 95°C, 40 s at 52°C, and 2 min at 72°C with additional extension time of 5 s per cycle. Samples were held for a final 10 min at 72°C to complete primer extensions, after which the samples were kept at 4°C until electrophoresis was performed on a 1% agarose gel prepared with TBE and stained with ethidium bromide.

PCR products were purified in a TAE 1% agarose gel, and desired bands were excised and cleaned of gel material using GELase (GEAgarose Gel-Digesting Preparation, Epicenter #G31100). Sequencing reactions were performed in a 10 μl final volume containing 2 μl of Big Dye (Big Dye Terminator Cycle sequencing Kit, ABI PRISM, Perkin-Elmer, Applied Biosystems), 2 μl of Big Dye buffer (5 μl 1 M MgCl₂, 200 μl 1 M Tris pH 9, and 795 μl sterile double-distilled water), 1 μl of 10 μM primer, and 5 μl of purified PCR product. Additional primers were used, in conjunction with PCR primers, for sequencing reactions on: the SSU (SR11R, SR7, SR7R, nssu634, nssu897R, and nssu1088R), LSU (LR3, LR3R, LR5, LR5R, LR6, LIC2028, and LR6R [5'-GGTAAAGC AGAAGTGGC-3']), and RPB2 (RPB2-908F, RPB2-1014R, RPB2-1554F, RPB2-1554R, RPB2-2473F, RPB2-2488F, RPB2-2492R, and RPB2-2568R) genes (see Supplementary material 2; Kauff and Lutzoni, 2002; Miadlikowska et al., 2002; and http://www.lutzonilab.net/pages/primer.shtml for references and sequences).

2.3. Phylogenetic analyses

2.3.1. Data sets, alignments and computing resources

Phylogenetic analyses were carried out on two data sets: an 83-taxon data set for which the SSU and LSU nrDNA and nuclear RPB2 gene sequences were obtained; and an 89-taxon data set restricted to SSU and LSU nrDNA. The various data sets with different taxon sampling and single or combined gene partitions will be referred to as: 83 SSU, 83 LSU, 83 RPB2, 83 SSU + LSU + RPB2, 89 SSU, 89 LSU, and 89 SSU + LSU.

Sequence fragments were assembled in Sequencher 3.0 (Gene Code Corporation, 1995) and sequences were aligned in MacClade 4.01 (Maddison and Maddison, 2001). Alignments of the SSU and LSU nrDNA sequences, and delimitation of ambiguously aligned regions, were performed according to Lutzoni et al. (2000) and using the secondary structure model (Kjer, 1995) of Saccharomyces cerevisiae (Saccharomyces; GenBank Accession No. U53879) provided by Cannone et al. (2002). The protein-coding gene RPB2 was aligned in MacClade using the option “nucleotides with amino acid colors” to facilitate manual alignment. For RPB2, ambiguously aligned regions were delimited manually (Lutzoni et al., 2000), taking into account the exchangeability of protein residues according to their chemical properties (Grantham, 1974).

Maximum parsimony and Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses were carried out on 6 Intrex clones (Athlon CPUs/1.6 GHz or 1.8 GHz) or on a computer farm of six Dell Optiplex GX260s (Pentium 4 processors/2.2 GHz). All MP, ML, and Bayesian bootstrap analyses were run on the IBM RS/6000 SP Unix systems at the North Carolina Supercomputing Center, which comprise 180 4-way 375 MHz Power3-II nodes. MP bootstrap analyses were run in parallel on 32 CPUs at a time. Each ML bootstrap pseudo-replicate was run on a separate CPU for a maximum of 14 CPUs simultaneously. Each Bayesian bootstrap analysis was run in parallel, with each of the four chains on one of the 4 CPUs of a given node.

2.3.2. Detecting topological incongruences among data partitions

To detect topological incongruences among partitions, we used a reciprocal 70% BP (Mason-Gamer and Kellogg, 1996) or a 95% PP threshold. Accordingly, topological conflicts were assumed to be significant if two different relationships (one monophyletic and the other non-monophyletic) for the same set of taxa were both supported with bootstrap values ≥ 70% or PP ≥ 95%. Bootstrap proportions were generated using neighbor-joining (NJ) non-parametric bootstrap (1000 replicates) with a maximum likelihood distance. Likelihood models were selected and parameters were estimated using the Hierarchical Likelihood Ratio Test.
(Modeltest 3.06; Posada and Crandall, 1998). Posterior probabilities used to detect conflicts among data partitions were generated using different Bayesian analysis approaches in MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). The third codon position of RPB2 showed saturation by multiple nucleotide substitutions for both transitions and transversions (saturation plots were obtained following Hackett, 1996; results not shown).

To assess potential conflict in phylogenetic signal for the third vs. first and second codons, we generated neighbor-joining trees with bootstrap support for each of the codon positions and used the 70% criterion to detect topological incongruence.

2.3.3. MP analyses

Weighted maximum parsimony analyses were run on both the 83 SSU + LSU + RPB2 and 89 SSU + LSU data sets. Unambiguously aligned portions of each data matrix were simultaneously subjected to multiple, symmetric step matrices. To build step matrices, changes between all character states (four nucleotides and gaps as a fifth character state) at each position of the unambiguously aligned regions were summarized using the “full detail” character status option in PAUP* 4.0b10 (Swofford, 2002). The program STMatrix 2.1 (written by S. Zoller and available at http://www.lutzonilab.net/pages/download.shtml) was used to calculate proportional frequency of changes, which were then converted into cost of changes using the negative natural logarithm (Felsenstein, 1981; Wheeler, 1990). Non-ambiguously aligned sites from the 83 SSU + LSU + RPB2 data set were subjected to five step matrices, including one for each of the ribosomal genes and one for each of the RPB2 codon positions. Frequency of changes were calculated independently for SSU and LSU. However, frequency of changes for each of the RPB2 step matrices (RPB2-1st, -2nd, and -3rd) were calculated over the total number of changes found in the entire gene. For one of the MP analyses, which incorporated proportional step matrices (STprop in the MP + I + A + STprop analyses; see Table 2), the frequency of changes for each of the five step matrices (SSU, LSU, RPB2-1st, -2nd, and -3rd) were calculated over the total number of changes found in the three-gene data set, making all five step matrices proportional to each other. All cost values were then divided by two so they would be similar to the substitution rates found in the SSU and LSU stepmatrices when calculated independently. The non-ambiguously aligned sites from the 89 SSU + LSU data set were subjected to either two independently calculated or two proportional step matrices, one for each of the ribosomal genes.

Ambiguously aligned regions were excluded from all analyses. However, some of these regions were re-coded and subjected to specific step matrices using INAASE v2.3b (Lutzoni et al., 2000), thus incorporating phylogenetic signal from these ambiguously aligned regions without violating positional homology (corresponding to MP analyses; see Table 2). We implemented a similar method to manually re-code ambiguously aligned regions of the RPB2 gene to satisfy codon constraints, which is not possible in the current version of INAASE. Ambiguous regions that were over 100 bp in length, highly variable (over 32 character states), or that showed major length variation among sequences of the same ambiguous region were re-coded into 23 characters using arc v1.5 (program written by F. Kauff and available at http://www.lutzonilab.net/pages/download.shtml#Ambiguous). Arc has two options that were used in MP analyses: a nucleotide option that takes into account the length of sequences for a given ambiguous region, the relative frequencies of bases and base pairs, and their distribution among sequences (Miadlikowska et al., 2003); and a protein option that takes into account the length of sequences in a given ambiguous region, and calculates the relative frequencies of the amino acids (corresponding to “A” of the MP analyses; see Table 2). Each of the 23 characters (character descriptions can be found in the arc manual) generated by arc-nucleotides were subjected to a specific weight: 1.00 for char. 1; 0.25 for chars. 2–5; 0.10 for chars. 6–15; and 0.50 for chars. 16–23. Weights for the 23 arc-protein characters were given as follows: 1.00 for char. 1; and 1 divided by the number of parsimony-informative characters for chars. 2–23 (uninformative arc-protein re-coded characters were excluded from the analyses). None of the introns present in the sequence alignments of RPB2 were coded into additional characters, except for the intron at position 2289 relative to Saccharomyces cerevisiae (GenBank Accession No. M15693) which was re-coded as present/absent and re-coded with the program arc-nucleotide.

For each MP analysis, a heuristic search was performed with 1000 random addition sequences (RAS), tree bisection-reconnection (TBR) swapping, and gaps coded as a fifth character state for the non-ambiguously aligned data. Bootstrap proportions for the 83 SSU + LSU + RPB2 data set were estimated with 512 pseudo-replicates and 48 RAS per pseudo-replicate. For the 89 SSU + LSU data set, 122–505 pseudo-replicates (Table 2) and 125–250 RAS per pseudo-replicate were used depending on the analysis done. The number of RAS per bootstrap pseudo-replicate was calculated by taking into account the number of times the shortest tree was found during the heuristic search using the original data set. All MP analyses were conducted with PAUP* 4.0b10 (Swofford, 2002).

2.3.4. Bayesian analyses

Bayesian posterior probabilities (PP) were computed with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) on both the 83 SSU + LSU + RPB2 and 89 SSU + LSU
Table 2
Summary of node support comparisons among different analyses of the 83 SSU + LSU + RPB2 and 89 SSU + LSU data sets

<table>
<thead>
<tr>
<th>Analysis type</th>
<th>83 SSU + LSU + RPB2 data set</th>
<th>89 SSU + LSU data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of replicates or sampled trees</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>No. of internodes recovered</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Total no. of resolved internodes in common with the reference</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Sum of all internodes support values</td>
<td>4517</td>
<td>4561</td>
</tr>
<tr>
<td>No. of significantly supported internodes</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>No. of significantly supported internodes in common with the reference</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>No. of significantly supported internodes lost</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>No. of significantly supported internodes gained</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The five-model Bayesian analysis of the 83 SSU + LSU + RPB2 data set (in bold) was used as a reference for all other analyses. Nodes were considered significant if support values were $\geq 70\%$ for MP, ML, and Bayesian bootstrap analyses, and $\geq 95\%$ for Bayesian analyses. To compare topologies derived from the 89-taxon data set with topologies inferring relationships among 83 taxa, the six internodes supporting the six additional taxa in the 89-taxon data set were ignored. “A”, addition of arc characters; “BP”, bootstrap proportions; “B-BP”, Bayesian bootstrap proportion; “I”, addition of INAASE characters; “ML”, maximum likelihood analysis; “MP”, maximum parsimony analysis; “PP”, posterior probabilities; “STprop”, implementation of proportional step matrices among partitions; “1mod” a single model of evolution; “5mod”, 5 models of evolution (one for each partition). A complete table with support values for each internode of all the analyses can be found in Supplementary material 3.
data sets. The 83 SSU + LSU + RPB2 data set was assumed to have five distinct partitions (SSU, LSU, RPB2-1st, -2nd, and -3rd codon positions) and was analyzed under a single model of evolution for the combined data set or using a model of evolution for each of the five partitions. The 89 SSU + LSU data set was considered to have two partitions (SSU and LSU) and was analyzed under two models of evolution, one for each partition. Models of evolution were selected in part with Modeltest 3.06 (Posada and Crandall, 1998).

The number of rate categories for the gamma distribution was estimated by comparing likelihood scores of a NJ tree under different numbers of gamma categories. If the likelihood score was improved by a minimum of 10 ln likelihood units, the number of rate categories for the gamma distribution was increased by one.

A preliminary Bayesian analysis was initiated using a random tree, four chains running simultaneously for 5,000,000 generations, and trees sampled every 100th generation. The tree with the best likelihood score was then used as a starting point for three separate runs of 5,000,000 generations each. After verifying that stationarity had been reached both in terms of likelihood scores and parameter estimation, the first 10,000 trees were discarded and a majority rule consensus tree was generated from the remaining 40,000 (post-burnin) trees from one of the second 5,000,000 generation runs chosen at random.

Following Douady et al. (2003a), Bayesian analyses were performed on 100 bootstrap 83 SSU + LSU + RPB2 data sets. These bootstrap data sets were assembled by generating 100 bootstrap pseudo-replicates for each of the five partitions (SSU, LSU, RPB2-1st, -2nd, and -3rd codon positions) using the program SEQBOOT 3.6a3 (Phylip; Felsenstein, 2002). One pseudo-replicate from each partition was then sampled without replacement to create a combined bootstrapped data set. Each of the 100 combined bootstrapped data sets was subjected to a Bayesian analysis as previously described, with the exception that each chain was only run for 2,000,000 generations. After discarding the first 10,000 trees, B-BP were obtained from a majority rule consensus of 1,000,000 trees (100 reps. × 10,000 trees) as suggested by Douady et al. (2003a).

2.3.5. ML bootstrap analysis

To have a more equitable comparison between Bayesian BP and ML-BP, ML analyses were performed on the same 100 combined SSU + LSU + RPB2 bootstrap data sets generated for the Bayesian bootstrap analysis. A model parameter summary was obtained from MrBayes 3.0b4 based on 40,000 post-burnin trees from a one-model Bayesian run on the combined 83 data set. Mean values for each of the parameters (sump option in MrBayes 3.0b4) were then used to fix the model of evolution for the ML search on the 100 bootstrap data sets (r[A < - > C] = 2.271974, r[A < - > G] = 6.398565, r[A < - > T] = 1.631732, r[C < - > G] = 1.119333, r[C < - > T] = 8.226414, r[G < - > T] = 1.000000, pi[A] = 0.228385, pi[C] = 0.261017, pi[G] = 0.266777, pi[T] = 0.243821, x = 0.316064, pinvar = 0.300560). The ML analyses on each bootstrapped data set were performed using heuristic searches in PAUP* with four RAS, TBR swapping, rearrangement limit of 10,000, reconnection limit of 8, and constant sites. ML bootstrap proportions were calculated by conducting a majority rule consensus analysis on the best trees from each of the 100-ML runs.

2.3.6. Reconstructing the evolution of polyspory

To reconstruct the origin and evolution of polyspory characterizing the Acarosporaceae, the number of 100 spores per ascus was taken as a limit in differentiating members of the Acarosporaceae sensu stricto, from other true polyspore species. This limit of over 100 could seem rather arbitrary, but was nevertheless used in this study, because various authors have used it to characterize the Acarosporaceae (Golubkova, 1988; Hafellner, 1995; Ozenda and Clauzade, 1970). Ancestral states for the number of ascospores per ascus were reconstructed on 20 representative nodes from the 83 SSU + LSU + RPB2 Bayesian phylogeny for the Pezizomycotina (Fig. 1). Nodes were selected based on their relative positions to true-polyspored species having over 100 spores per ascus (i.e., Acarosporaceae s. l.), and their potential ability to resolve the evolution of this type of polyspory. Ancestral states for these nodes were reconstructed on 4000 trees drawn randomly from the 40,000-tree pool sampled during the 83 SSU + LSU + RPB2 Bayesian analysis. Two character states were considered: 0 = octospory, true polyspory with fewer than 100 spores per ascus (TP < 100), and polyspory resulting from budding of original spores or fragmentation of septate spores (apparent polyspory); and 1 = true polyspory with more than 100 spores per ascus (TP < 100).

Five different reconstructions (A, B, C, D, and E) were implemented to accommodate three taxa thought to be problematic in character state coding, and to evaluate the effect of character coding on ancestral state reconstruction. Acarospora macropora and Glypholeon scabra (names followed by a pound sign in Fig. 1), both members of the Acarosporaceae s. str., have fewer than 100 spores per ascus. Therefore, they were coded either as 0 for having TP < 100, or as 1 because other morphological characters placed them in the Acarosporaceae and the low number of spores could be due to a reduction of the number of post-meiotic mitoses. The octosporous basidiomycete outgroup, Athelia bombicina, has basidiospores instead of ascospores and, therefore, it could be argued that basidiospores are not homologous to ascospores. Consequently, Athelia...
Fig. 1. Phylogenetic placement of the family Acarosporaceae within the Ascomycota based on a Bayesian MCMC analysis of the combined SSU nrDNA, LSU nrDNA, and RPB2 nDNA data set for 82 species of the Ascomycota, including 14 species from the Acarosporomycetidae/Acarosporaceae, and one basidiomycete species used as outgroup (i.e., 83 SSU + LSU + RPB2). The phylogram represents the majority rule consensus tree of 40,000 post-burnin trees sampled by the B-MCMC and has an arithmetic mean likelihood value of 94832.97. Lengths for each branch were averaged over all trees (sumt option in MrBayes v3.0b4). Numbers above internodes that are before the backslash are B-MCMC posterior probabilities (PP). Values above internodes that are after the backslash are Bayesian bootstrap proportions (B-BP). Values below internodes are ML bootstrap proportions (ML-BP). Internal branches for which the Bayesian posterior probability was ≥95% and the likelihood bootstrap was ≥70% are shown as a thicker line. If PP and B-BP were ≥95 and ≥70%, respectively, and the ML-BP was <70%, the internode and associated support values are circled. The grey box delimits the Acarosporomycetidae subclass nov. Names followed by an asterisk are true-polyspored species with over 100 spores per ascus. Names followed by a pound sign are true-polyspored species with fewer than 100 spores per ascus. Supra-generic taxon names follow, in part, classifications by Eriksson et al. (2003); Kauff and Lutzoni (2002), Kirk et al. (2001), Miadlikowska and Lutzoni (2004), and Taylor et al. (in press). Names marked with a black triangle correspond to a class or a subclass newly introduced in this study. Supra-generic taxon names with a question mark indicate that the placement of the corresponding species in that supra-generic taxon cannot be confirmed by our results. The dashed line with a question mark indicates an alternative and preferable circumscription of the Lecanoromycetes, but this option could not be adopted because of the low support value at the preferred node.
*bombacina* was coded either as 0 (equivalent to eight ascospores, the expected ancestral state for ascomycetes) for three of the reconstructions (A, B, and D) or removed from the analyses during reconstructions C and E (taxon manually removed from the 4000 Bayesian trees). All other members of the Acarosporaceae s. l. (taxa names followed by a star in Fig. 1) were coded as character state 1, and the remaining ascomycetes were coded as character state 0. These ascomycete species have between 1 and 8 spores per ascus, except for *Peltula obscurans*, which has 80 spores per ascus, and some anamorph species, which are not known to produce meiospores. Ancestral states were reconstructed using maximum likelihood as the optimality criterion (Pagel, 1999) with the “trace character over trees” option in Mesquite 1.0 (Maddison and Maddison, 2003). An asymmetrical 2-parameter Markov k-state model allowing different rates of gains and losses was selected to reconstruct ancestral states. Rates of changes were estimated from the data using both character repartition and tree. An ancestral state at a given node was considered significant and preferred over the other if its likelihood value was higher by at least two log units than the likelihood value of the other ancestral state (likelihood decision threshold values [T] set to two by default in Mesquite, as suggested by Pagel at [http://sapc34.rdg.ac.uk/meade/Mark/files/DiscreteManual.pdf](http://sapc34.rdg.ac.uk/meade/Mark/files/DiscreteManual.pdf)).

### 3. Results

#### 3.1. Alignment

A summary of alignment sites used in the different analyses is shown in Table 3. In the 83 SSU + LSU + *RPB2* data set, SSU and LSU together contributed to 77.9% of the total alignment length; nevertheless, the *RPB2* partition alone accounted for 58.3% of the variable sites included in the Bayesian and ML analyses. This reflects the presence of numerous group I and spliceosomal introns (Bhattacharya et al., 2002, 2000) at 31 splicing sites of the SSU and LSU. In contrast, *RPB2* has only small spliceosomal introns at six splicing sites. Most of the variation in the *RPB2* gene occurs at the 3rd codon position, with 49.7% of the variable sites relative to the 29.9 and 20.4% at the 1st and 2nd positions, respectively. By considering a non-ambiguously aligned gap as a fifth character state, and by re-coding ambiguously aligned regions as well as one spliceosomal intron in the MP analyses, we gained 457 (=163.3 down-weighted) additional parsimony-informative characters that were not included in the Bayesian analyses. When gaps were considered as a fifth character state for unambiguously aligned sites, and ambiguously aligned regions were re-coded, the number of parsimony-informative sites included in the

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Summary of data sets used for MP, Bayesian, and ML analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSU</td>
<td>LSU</td>
</tr>
<tr>
<td>No. of sites in alignment</td>
<td>5043</td>
</tr>
<tr>
<td>No. of excluded sites</td>
<td>4061</td>
</tr>
<tr>
<td>No. of ambiguously aligned regions</td>
<td>22 (179)</td>
</tr>
<tr>
<td>No. of introns with introns</td>
<td>22 (19, 3)</td>
</tr>
<tr>
<td>No. of regions with introns (INAASE, arc)</td>
<td>15 (3882)</td>
</tr>
<tr>
<td>Total no. of sites analyzed</td>
<td>982</td>
</tr>
<tr>
<td>No. of constant sites</td>
<td>586</td>
</tr>
<tr>
<td>No. of variable sites</td>
<td>396</td>
</tr>
<tr>
<td>No. of parsimony-informative sites_</td>
<td>242</td>
</tr>
</tbody>
</table>

> Excluded sites comprise ambiguously aligned regions (row 3), as well as spliceosomal and group I introns (row 5).
89 SSU + LSU MP analyses increased by 342 (=137.7
down-weighted) characters compared to the Bayesian
and ML analyses.

3.2. Topological incongruences among data partitions

Preliminary Bayesian analyses using different set-
tings to assess topological incongruences among data
partitions of the two- and three-gene data sets, revealed
well-supported conflicts among data partitions, unexpected
relationships among taxa or clades, or a lack of stationarity after five million Bayesian generations. These Bayesian analyses may have over-estimated
support for clades, especially in the case of very short
internodes (see Alfaro et al., 2003), or may have been
sensitive to small model misspecifications, causing an
incorrect topology to be preferred (Buckley, 2002;
Buckley et al., 2002; Huelsenbeck et al., 2002; Waddell
et al., 2001). It is also possible that single gene analyses
included too few characters to generate reliable esti-
mates of posterior probabilities for such a large
number of taxa. To avoid this large number of false
positive conflicts generated by MrBayes, we tested for
topological incongruence using an NJ (with ML dis-
tance) bootstrap approach. This conservative approach
seems to generate fewer false positives when conduct-
ing large-scale phylogenetic studies (MP or ML boot-
strap analyses being computationally too time
consuming for large-scale phylogenies). Using the re-
ciprocal 70% BP criterion, we detected a lower number
of potential conflicts compared to the Bayesian analy-
sis, and these conflicts were all found to result from
problematic contaminated sequences (see also Lutzoni
et al., 2004). As a result, we excluded two highly sup-
ported conflicting taxa from the three-gene data set, for
a final data set of 83 taxa, and three highly supported
conflicting taxa from the two-gene data set, for a final
data set of 89 taxa.

By comparing bootstrap NJ tree topologies obtained
for the 83 SSU, 83 LSU, and 83 RPB2 partitions, we
found two poorly supported conflicts. Despite these
conflicts, we combined the three data sets because two of
the reciprocal support values (70 and 72%, respectively)
were at the threshold of being considered significant,
which does not make a strong case for conflicting to-
polgies. A 70% bootstrap proportion is often close to,
but does not guarantee, 95% accuracy (Alfaro et al.,
2003; Hillis and Bull, 1993). The bias between bootstrap
proportions and phylogenetic accuracy depends upon
numerous variables, such as the method of phylogenetic
inference, inferred rate of change, number of taxa and
characters, and independence of characters and, there-
fore, can vary from study to study. For example, under
very high rates of internodal change, or highly unequal
rates of change among taxa, bootstrap proportions
>50% may overestimate accuracy (Hillis and Bull,
1993). Comparison of NJ bootstrap proportion did not
show topological conflict between RPB2-1st, -2nd, and
-3rd codon positions. Therefore, we concluded that sat-
uration at the 3rd codon position would not negatively
affect phylogenetic accuracy when combined with the
other partitions. Using the same criterion, no conflict
was detected between the 89 SSU and 89 LSU data sets,
such that these two data sets were combined.

3.3. Comparison of methods to reconstruct phylogenies

A summary of the analyses performed in this study is
presented in Table 2. Bayesian posterior probabilities
based on the analysis of the 83 SSU + LSU + RPB2 data
set using five models of evolution (Bayesian 5mod-PP)
were used as a reference for comparison of internode
support. We observed a decrease in number of inter-
nodes recovered, sum of all internode support values,
and number of significantly supported internodes as we
went from a five-model Bayesian analysis, to a one-
model Bayesian analysis, a five-model Bayesian boot-
strapped analysis, and a one-model ML analysis (Table
2). Relative to the one-model ML analysis, the one-
model Bayesian analysis appears more powerful in
recovering well-supported internodes (63 vs. 54). How-
ever, the ML analysis recovered a topology more similar
to the five-model Bayesian analysis, as its total number
of resolved internodes in common with the reference is
higher than for the one-model Bayesian analysis (76 vs.
72).

The number of internodes with support values ≥70% for
parsimony analyses generally increased with the addi-
tion of INAASE (I) and arc (A) characters, and with the
implementation of proportional step matrices (STprop)
on non-ambiguously aligned regions (MP + I + A +
STprop). However, the increase in well-supported inter-
nodes on the 89 SSU + LSU MP + I + A + STprop tree
was accompanied by a decrease in the sum of all internode
support values (4029 vs. 4069) and the total number of
resolved internodes in common with the reference phylo-
geny (51 vs. 55), relative to the 89 MP + I + A
analysis (Table 2). The addition of proportional step
matrices to the INAASE and arc characters in the MP
analyses of the 89 SSU + LSU data set was not advan-
tageous, but did prove itself in MP analyses of the 83
SSU + LSU + RPB2 data set.

The Bayesian five-model consensus tree for the
83 SSU + LSU + RPB2 data set revealed 68 signifi-
cantly supported internodes (PP ≥95%) vs. only 46
(BP ≥70%) in the most parsimonious tree obtained with
the MP + I + A + STprop analysis. Only two notable
differences (79 and 82% ML-BP conflict) appeared be-
tween the two topologies. The Bayesian two-model
analysis (Bayesian 2mod) on the 89 SSU + LSU data set
generated two additional significantly supported inter-
nodes compared to the MP + I + A analysis; however, the
3.4. Comparison of methods for assessing phylogenetic confidence (PP, B-BP, and ML-BP)

A total of 79 internodes were recovered from the B-MCMC analysis of the 83-taxon combined data set (50% majority rule consensus) out of 80 potential internodes for a totally resolved unrooted topology. Of these 79 internodes, 68 were statistically significant (i.e., PP > 95%), 59 had B-BP values ≥ 70%, and 54 had ML-BP values ≥ 70% (Table 2). All branches supported with ML-BP ≥ 70% were also supported by PP ≥ 95%, whereas the reciprocal was not true (Supplementary material 4A). This result agrees with other phylogenetic studies on data sets containing large numbers of taxa (62 taxa in Kauff and Lutzoni, 2002; 78 in Leaché and Reeder, 2002). However, Kauff and Lutzoni (2002) also recorded, on a data set of 28 taxa, that almost all branches with PP ≥ 95% also had ML-BP > 70%, and that PP and ML-BP seemed to be correlated. These results were also found by Whittingham et al. (2002) on a data set of 31 taxa. For cases in which support values were unequal for internodes present in our 83-taxon Bayesian five-model tree (Fig. 1), the PP was always greatest, while ML-BP had the lowest support values on 34 internodes vs. 13 for B-BP. However, when B-BP values were the lowest, they were not very different from the ML-BP values, such that overall, B-BP values are intermediary between PP and ML-BP (Supplementary material 3, 4A). A comparison of PP and B-BP with ML-BP support values (Supplementary material 4A) corresponds with findings obtained by Douady et al. (2003a) on several empirical data sets. The ML-BP values are more strongly correlated with B-BP values (r = 0.852) than with PP values (r = 0.296). B-BP values and PP values also appear correlated (r = 0.671). A comparison of the differences between ML-BP and Bayesian PP for a given internode and the length of that internode shows that the largest discrepancies between the two measures generally occur on shorter internodes (Supplementary material 4B).

3.5. Contribution of RPB2 to resolving ascomycete relationships

In general, all major Ascomycota lineages presented by Kauff and Lutzoni (2002), Lutzoni et al. (2001), and Miadlikowska and Lutzoni (2004), based on combined nuclear ribosomal SSU and LSU, were recovered in the present study, and their statistical significance (PP ≥ 95%, BP ≥ 70%) maintained (Fig. 1). These lineages include the Saccharomycotina (100% for all three types of support: PP, B-BP, and ML-BP); the Pezizomycotina (100% for PP, B-BP, and ML-BP); the node after the split of the Pezizomycetes (100% PP and B-BP and 99% ML-BP); the Sordariomycetes (100% PP, 91% B-BP, and 74% ML-BP); the Lichinomycetes (100% for PP, B-BP, and ML-BP); the Leotiomycetes (100% PP and 82% B-BP); and finally the Acharosporomycetidae subclass. nov. (100% for PP, B-BP, and ML-BP).

On the other hand, our study presents differences relative to recent phylogenetic studies (Kauff and Lutzoni, 2002; Liu and Hall, 2004; Liu et al., 1999; Lücking et al., 2004; Lumbsch et al., 2001, 2002, 2004; Lutzoni et al., 2001, 2004; Miadlikowska and Lutzoni, 2004). In our Bayesian five-model 83 SSU + LSU + RPB2 tree (Fig. 1), the Pezizomyces form a well-supported paraphyletic group (98% PP, 76% ML-BP), with Peziza quelepidotia part of a distinct lineage, and Gyronmitra esculenta and Morechella esculenta sister to the rest of the Pezizomyces. However, this paraphyletic grouping could be due to long-branch attraction (Felsenstein, 1978) associated with a poor sampling of the Pezizomyces. A phylogenetic analysis of a combined nuclear SSU + LSU data set of 558 species representing all traditionally recognized fungal phyla (Ascomycota, Basidiomycota, Chytridomycota, and Zygomycota) and the Glomeromycota, and which included 21 taxa classified within the Pezizomyces, revealed this class of fungi as being monophyletic with a significant PP of 96% and an NJ (with ML distance) bootstrap value of 70% (Lutzoni et al., 2004). Our delimitation of the well-supported Sordariomycetes (100% PP, 91% B-BP, and 74% ML-BP), including Arthoniomycetidae, Dothideomycetidae, and Sordariomycetidae, differs from Liu and Hall (2004) and Lumbsch et al. (2002), who keep the Sordariomycetes and Dothideomycetes as two separate classes that do not group together. The Leotiomycetes, monophyletic in other studies (Kauff and Lutzoni, 2002; Liu and Hall, 2004; Liu et al., 1999; Lumbsch et al., 2001; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004) was found to be paraphyletic in our analyses. In particular, we found that Cudonia circinans and Rhytisma acerinum form a monophyletic group (Leotiomycetes 1 in Fig. 1) sister to the Sordariomycetes, with 97% PP support, but only 68% B-BP, and <50% ML-BP. Trichoglossum hirsutum, the other member of the Leotiomycetes included in our study (Leotiomycetes 2 in Fig. 1), was
found to be sister to the Lecanoromycetes, but all three support values for this relationship are below 55%. Therefore, relationships among members of the Leotiomycetes and their placement in the euascomycetes remain highly uncertain based on this limited sampling.

Our delimitation of the Eurotiomycetidae differs from that of Liu and Hall (2004), who found the Chaetothyriales (including a member of the order Verrucariales) sister to the Pleosporales + Dothideales clade (corresponding to the Dothidiomycetidae in our Fig. 1), while the rest of the Eurotiomycetes grouped with the sordariomycetes (Sordariomycetidae in Fig. 1) + Heotiales + lichenized fungi. Our three-gene phylogeny shows that the four orders Chaetothyriales, Eurotiiales, Pyrenulales, and Verrucariales form a monophyletic group with 100% PP, 89% B-BP, and 62% ML-BP. In addition, our study excludes the Umbilicariaceae from the Eurotiomycetidae as opposed to previous studies (Kauff and Lutzoni, 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004), although this placement within the Eurotiomycetidae was mostly not well supported in these studies. The placement of the Umbilicariaceae remains uncertain within the Lecanoromycetes. However, this family appears closely related to the Fusicaceae (100% PP, 88% B-BP, and 70% ML-BP, Fig. 1). The Pertusariaceae–Icmadophilaceae clade (100% PP and B-BP, and 99% ML-BP) is here shown to be part of the first split at the base of the Ostropomycetidae as revealed by the Bayesian analysis of the 83 SSU + LSU + RPB data set (Fig. 1). Sarcosagium campestre forms a monophyletic group with Biatoridium (76% MP-BP); however, the placement of this clade remains uncertain. Our MP + I + A analysis does not allow us to elucidate, with high phylogenetic confidence, the systematic position of the polyspored species Sarea resinae. According to the anatomical work of Bellemère (1994), this species remains of uncertain position, whereas, Eriksson et al. (2004) placed it in the Agyriales. Scoliciosporum umbrinum did not group with Strangospora as was expected (Hafellner, 1995), however, it seems to be a member of the Lecanoromycetidae, more specifically of the Lecanorales s. str., where it is currently classified (Eriksson et al., 2004). As expected, Verrucaria groups with Dermatocarpon (99% MP-BP).

3.6. Phylogenetic placement of Maronea, Sarcosagium, Sarea, Scoliciosporum, Thelocarpella, and Verrucaria based on combined nuclear SSU + LSU nrDNA

A single most parsimonious tree of 10,260.91 steps was obtained from the MP + I + A search (Fig. 2). The topology of this tree is very similar to the Bayesian tree shown in Fig. 1. Except for the phylogenetic placement of Microascus and Diaportha (Sordariomycetidae), none of the discrepancies between the two topologies were supported by MP-BP ≥ 70% in the MP + I + A tree.

Our MP + I + A tree shows that Thelocarpella gordensis is part of the Acarosporomycetidae (90% MP-BP) and is closely related to Polysporina cf. simplex (85% MP-BP). Maronea constans groups with Maronea chilensis and Fuscidea lygaea (100% MP-BP; Fig. 2), which confirms the phylogenetic placement of Maronea within the family Fusciaceae as revealed by the Bayesian analysis of the 83 SSU + LSU + RPB2 data set (Fig. 1). Sarcosagium campestre forms a monophyletic group with Biatoridium (76% MP-BP); however, the placement of this clade remains uncertain. Our MP + I + A analysis does not allow us to elucidate, with high phylogenetic confidence, the systematic position of the polyspored species Sarea resinae. According to the anatomical work of Bellemère (1994), this species remains of uncertain position, whereas, Eriksson et al. (2004) placed it in the Agyriales. Scoliciosporum umbrinum did not group with Strangospora as was expected (Hafellner, 1995), however, it seems to be a member of the Lecanoromycetidae, more specifically of the Lecanorales s. str., where it is currently classified (Eriksson et al., 2004). As expected, Verrucaria groups with Dermatocarpon (99% MP-BP).

3.7. Reconstructing the evolution of polyspory within the Pezizomycotina

The evolution of polyspory was reconstructed for 20 crucial nodes within the Pezizomycotina (Fig. 3). The ancestral state for true polyspory with over 100 spores per ascus (TP > 100) was found to be significant (i.e., T > 2) on four nodes (5, 16, 17, and 20) in 100% of the trees sampled for all five reconstructions (Fig. 3). Assignment of an ancestral state for all other nodes varied among the different reconstructions (A through E). However, for nodes 9 and 14, character state “0” was always present in more that 50% of the trees sampled for all five reconstructions. Except for nodes 5, 16, 17, and 20, there is a general tendency to go from ancestral character state 0 (octospory, TP < 100, or polyspory from budding and fragmentation), to equivocal reconstruction (non-significant for either state), to an increased number of trees having ancestral character state 1 (TP > 100) at a given node. For example, the ancestral character state on node 4, reconstruction A, was
Fig. 2. Phylogenetic circumscription of the Acarosporaceae, and placement of Sarcosagium, Sarea, Scoliciosporum, Thelocarpella, and Verrucaria within the Pezizomycotina (euscomycetes) based on the combined SSU and LSU nrDNA data set for 88 species of the Ascomycota, including 15 species from the Acarosporomycetidae/Acarosporaceae, and one basidiomycete species used as outgroup (i.e., 89 SSU + LSU). The phylogram represents the single most parsimonious tree (10,260.91 steps, CI = 0.3497, CI excluding uninformative characters = 0.3127, RI = 0.4474) when accommodating signal from ambiguously aligned gap-rich regions (INAASE and arc characters, i.e., MP + I + A). Parsimony bootstrap values are shown above internal branches when P ≥ 50%. If the parsimony bootstrap support was P ≥ 70%, internal branches are shown with thicker lines. The grey box represents our delimitation of the Acarosporomycetidae/Acarosporaceae for taxa included in this study. Names followed by an asterisk are true-polyspored species with over 100 spores per ascus. Names followed by a pound sign are true-polyspored species with fewer than 100 spores per ascus. Species names in bold correspond to the six additional taxa which were not present in the 83-taxon data set. Supra-generic taxon names follow, in part, classifications by Eriksson et al. (2004), Kauff and Lutzoni (2002), Kirk et al. (2001), Miadlikowska and Lutzoni (2004), and Taylor et al. (in press). Supra-generic taxon names with a question mark indicate that the placement of the corresponding species in that supra-generic taxon cannot be confirmed by our results.
significant for state 0, became equivocal in reconstructions B, C, and D, then changed to state 1 in a majority of trees (53%) in reconstruction E. These results show that, even if the analyses are based on a large number of trees and taxa, changing the character coding of a few taxa significantly influences ancestral state reconstruction (see also Omland, 1999). Furthermore, changing character state coding of taxa at the tip of the tree (e.g., *A. macrospora* and *G. scabra*) has more influence on our ancestral state reconstructions than do changes to the coding of taxa at the root of the tree (e.g., *A. bombacina*; see reconstructions B and D vs. B and C).

4. Discussion

4.1. Maximum parsimony incorporating ambiguously aligned regions vs. Bayesian methods restricted to alignable regions

In our experience, the inclusion of INAASE and arc re-coded characters into the MP analysis (MP + I + A) of the SSU and LSU appeared to be advantageous in reconstructing the phylogeny of the Ascomycota. This is shown by an increase of the number of supported internodes, in both the 83-and 89-taxon combined data sets,
compared to a weighted parsimony analysis restricted to alignable regions (Table 2). The implementation of proportional step matrices (STprop) on the non-ambiguously aligned regions during MP + 1 + A + STprop analyses also increased the number of supported internodes for both data sets. The slight increase of supported internodes on the 89 SSU + LSU data set was, however, associated with a decrease in the sum of all support values and in the number of internodes in common with the reference analysis (Table 2). Applying proportional step matrices to the SSU and LSU data did not give more resolution, but was helpful when RP2 was included in the analyses. RP2 varies markedly in substitution rate among codon positions, such that proportional step matrices prevent overweighting substitutions at the 3rd codon position compared to the 1st and 2nd codon positions, or of the SSU and LSU partitions.

The Bayesian method, considered to be more efficient than other phylogenetic methods, did not have greater power in resolving the phylogeny of the 89 SSU + LSU data set than did MP + 1 + A (Table 2). Both SSU and LSU evolve relatively slowly, and without phylogenetic signal from ambiguously aligned regions, there was insufficient variation even for Bayesian analyses to resolve relationships for these 89 taxa with high phylogenetic confidence. In addition, some phylogenetic relationships found on the 89-taxon Bayesian tree were puzzling (see Section 3.3). A possible explanation is that the Bayesian method is more prone to give high support for incorrect relationships when the amount of phylogenetic signal is low (very short branches; see Alfaro et al., 2003). When RP2 data were included, resolution and support values resulting from the Bayesian analysis improved, reflecting an increase in phylogenetic signal, whereas, MP analyses, even with re-coded characters from the ambiguous regions, did very poorly (Table 2). The implementation of five models of evolution during Bayesian analyses of the 83 SSU + LSU + RP2 data set gave greater support than when using a single model for all three genes, likely reflecting differences in model of evolution between RP2 and the rDNA genes. Finally, bootstrapped Bayesian analysis and ML analysis did not perform as well as did a conventional Bayesian analysis (fewer supported internodes recovered, Table 2), but did show greater resolution and support than any MP analyses. ML analyses on large data sets are computationally expensive, and current programs can implement only one model of evolution at this time. However, bootstrapped Bayesian analyses are faster, and the current version of MrBayes allows the use of multiple models of evolution. In addition, bootstrapped Bayesian analysis is expected to be less prone to providing high bootstrap proportions for wrong internodes (Douady et al., 2003a) because it is less likely to attribute high support to the same incorrect internodes on each of the 100 bootstrapped data sets. However, further investigations are needed. Overall, when more molecular characters are added to a given set of taxa, Bayesian analysis using different models of evolution is likely to become more efficient than recovering phylogenetic signal from ambiguously aligned regions using a parsimony-based approach such as INAAASE (Lutzoni et al., 2000), arc (Miadlikowska et al., 2003) and POY (Wheeler et al., 2003). In turn, bootstrapped Bayesian analyses seem to give the best estimate of confidence in internode support (see also Section 4.2), but this needs to be confirmed with simulation studies.

### 4.2. Interpretation of PP, B-BP, and ML-BP in assessing phylogenetic confidence

Robust support values are essential for a meaningful interpretation of phylogenetic relationships. Most recent studies evaluate the reliability of internodes with non-parametric bootstrap proportions (Felsenstein, 1985) and Bayesian posterior probabilities (Huelsenbeck and Ronquist, 2001; Larget and Simon, 1999; Li, 1996; Mau, 1996; Rannala and Yang, 1996). However, it is not yet clear how PP relates to BP, and which of these two measures more accurately estimates phylogenetic confidence. Some authors suggest both types of measures to be equivalent (Cummings et al., 2003; Efron et al., 1996; Huelsenbeck et al., 2001; Larget and Simon, 1999), while others, based on empirical and simulated data, found discrepancies and/or lack of correlation (Alfaro et al., 2003; Buckley et al., 2002; Douady et al., 2003a,b; Erixon et al., 2003; Kauff and Lutzoni, 2002; Leaché and Reeder, 2002; Suzuki et al., 2002; Whittingham et al., 2002; Wilcox et al., 2002).

Our 83 SSU + LSU + RP2 phylogeny also showed high discrepancies between PP and ML-BP. We found that ML bootstrap support values were always lower or, at the most, equal to the corresponding PP, that more internodes were supported by PP \( \geq 95\% \) than by ML-BP \( \geq 70\% \), and that there was little correlation between the two types of support values (\( r = 0.296 \)). Several hypotheses have been put forward to explain such discrepancies. First, the methodologies used to calculate phylogenetic uncertainty differ (Alfaro et al., 2003; Erixon et al., 2003; Huelsenbeck et al., 2002) and, therefore, are expected to give different results. Second, the calculation of bootstrap proportions is thought to be biased (Hillis and Bull, 1993). However, the only method proposed to correct for this bias (Efron et al., 1996) is computationally too expensive to be easily implemented. Third, Bayesian inference appears to be more sensitive to model misspecification than are other methods (Buckley, 2002; Buckley et al., 2002; Huelsenbeck et al., 2002; Waddell et al., 2001), leading to overconfidence in topologies and internode support. This is even more problematic when dealing with higher numbers of taxa,
because evolutionary models become more complex and more difficult to implement (Buckley, 2002; Buckley et al., 2002; Huelsenbeck et al., 2002). Finally, the number of short false internodes with high posterior probabilities obtained in Bayesian analyses can be expected to increase when taxa are added without increasing the number of characters or sites.

In addition to these general causes of potential discrepancies between PP and ML-BP, more specific causes apply to our 83-taxon data set. First, we applied five models of evolution (one per partition) for the Bayesian analysis, during which parameters were optimized, while the ML analysis was subjected to a single model of evolution with fixed parameters. We observed a decrease in number of well-supported internodes and the sum of all internode support values, when implementing a Bayesian analysis with a single model of evolution on the original 83-taxon data set compared to the five-model analysis (Table 2). We would expect these numbers to drop even more if we used bootstrapped data sets. Second, parameters used to run the ML bootstrap analysis were estimated as accurately as possible on the original data set, nevertheless, stochastic errors associated with our analysis probably increased due to the limited number of random additions sequences, bootstrap pseudo-replicates, and topological rearrangements used. Another factor that could bias the comparison of bootstrap with Bayesian PP is that ML bootstrap analyses can be performed using parameters estimated from the original data set vs. re-estimating the parameters for each pseudo-replicate. However, Cummings et al. (2003) indicated that the mean values for the two different bootstrap procedures should not differ significantly, with the exception that re-estimating parameters on each pseudo-replicate requires more computational time.

A major concern in assessing internode support is the tendency of Bayesian methods (as implemented in MrBayes v3.0b4; Huelsenbeck and Ronquist, 2001) to give high support values to more incorrect internodes than would bootstrap methods (Alfaro et al., 2003; Douady et al., 2003a; Erixon et al., 2003; Suzuki et al., 2002), especially when short internodes are involved. Indeed, various studies have shown that the largest discrepancies between BP and PP occur on very short internal branches (Alfaro et al., 2003; Kauff and Lutzoni, 2002; Supplementary material 4B), and that PPs are more variable for very short internodes than are PPs estimated on longer internal branches, thus suggesting that the accuracy of PP may be data set specific (Alfaro et al., 2003). The fact that Bayesian inferences are more prone to this type of error could be the source of topological conflicts, as observed by Buckley et al. (2002), Douady et al. (2003b), and in our test for incongruence between data partitions using the PP > 95% criterion (see Section 3.2). These observations imply that PP as a sole measure of support might be misleading. Lewis and Holder (2003) attribute the increased level of this type of errors for Bayesian inference to the fact that MrBayes does not allow for branch lengths of zero. As a result, the topology will be resolved even in the absence of any data to support certain internodes, which could result in high PP on a very short branch. One way to decrease this type of error would be to allow polytomies in trees sampled with Bayesian MCMC (Lewis and Holder, 2003). Another way, proposed by Douady et al. (2003a), would be to conduct Bayesian analyses on bootstrapped data sets.

We found that correlation between B-BP and PP is higher than between ML-BP and PP, probably because we used the same five models of evolution to generate PP on the original data set as on the 100 bootstrapped data sets. However, according to Douady et al. (2003a), differences that still exist between B-BP and PP can be explained by the fact that B-BP, being more conservative, might be less prone to give high support values for wrong internodes. By using these B-BP support values, Douady et al. (2003a,b) were able to eliminate topological conflicts that existed between data partitions, and B-BP values gave higher support to true internodes than did ML-BP values. In our analysis, B-BP revealed six additional internodes with significant support (i.e., >70%), which had over 95% PP but were supported by ML-BP < 70% (circled internodes in Fig. 1).

Douady et al. (2003a) showed that the strong correlation between ML-BP and B-BP was not due to chance alone. Our lower level of correlation between these two measures of support was probably due to the use of a single model of evolution to obtain ML-BP values, vs. five models to generate B-BP values (Table 2). The use of five models of evolution on our three-gene data set is probably more realistic in recovering the true phylogeny and, therefore, our B-BP values may be more trustworthy for assessing clade support than our one-model ML-BP values. B-BP values cannot be interpreted as posterior probabilities, but rather as bootstrap proportions because B-BPs are derived from non-parametric bootstrapped data sets. The strong correlation between B-BP and ML-BP (r = 0.852, Supplementary material 4A) supports the interpretation of these B-BP values as bootstrap values in general. Until Bayesian MCMC methods can handle polytomies (Lewis and Holder, 2003), B-BP seems to be a good compromise between the higher accuracy of PP, but with higher numbers of false internodes with PP > 95%, and the lower, accuracy of ML-BP, but with lower numbers of wrong clades with support values > 70%.

4.3. Phylogenetic placement and circumscription of the Acarosporaceae

As shown in previous studies (Kauff and Lutzoni, 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni,
2004), the family Acarosporaceae forms an independent lineage outside the core Lecanoromycetidae and should be excluded from the Lecanorales sensu Eriksson et al. (2004). The subclass Acarosporomycetidae was first introduced by Miadlikowska and Lutzoni (2004) and Taylor et al. (in press) to accommodate the Acarosporaceae at the same level as the other three major lineages of the class Lecanoromycetes. We here propose to formally recognize this new subclass that includes the single family Acarosporaceae. The family Hymeneliaceae, which was included in the Acarosporinae together with Acarosporaceae (Eriksson et al., 2004; Poelt, 1973; Rambold and Triebel, 1992), does not belong to the Acarosporomycetidae. Its representative in our tree, Ionaspis lacustris, is nested within the Ostropomycetidae (Fig. 1), a placement already shown by Bhattacharya et al. (2000) with Hymenelia epulotica. Therefore, the suborder Acarosporinae is no longer relevant, and should be abandoned.

Subclass. Acarosporomycetidae V. Reeb, Lutzoni and C. Roux subclass. nov.

Diagnosis. Thallus crustaceus, squamulosus, raro foliaceo-umbilicatus; photosymbiota chlorococcoidae; ascomata immersa vel sessilia, in forma apotheciorum (cryptolecanorinorum, lecanorinorum vel lecideinorum, rarius biatorinorum vel pseudolecanorinorum), raro in forma peritheciorum; ascosporeae generaliter plus quam centum in ascis, simplices et incolores, halo expertes; paraphyses s. l. mediocriter vel infirme ramoso-anastomosae; ascii bitunicati, officio unitunicati, cum tholo non-amyloidei vel satis infirme amyloidei, cum oculari camera.

Thallus crustose, squamulose, rarely foliose-umbilicate; photobiont chlorococcoid; ascomata immersed or sessile, in form of apothecia (cryptolecanorine, lecanorine, or lecideine, more rarely biatorine or pseudolecanorine), rarely in form of perithecia; ascospores generally more than a 100 per ascus, simple and colorless, without halo; paraphyses s. l. moderately or slightly branched-anastomosed; ascii bitunicate, functionally uniloculate, non-amyloid or slightly amyloid tholus, presence of ocular chamber.

Circumscription. See Fig. 1.

Type family. Acarosporaceae Zahlbr.; type genus—Acarospora V. A. Massal.

Previous phylogenies failed to reveal relationships among the four subphyla of the Lecanoromycetes (Ekman and Tonsberg, 2002; Kauff and Lutzoni, 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004). By adding a protein-coding gene to the analyses, we were able to show that the Acarosporomycetidae is sister to the well-supported Ostropomycetidae + Lecanoromycetidae clade, with the Eurotiomycetidae at the base of the other three subclasses. However, we are still lacking support for some of the relationships among the Lecanoromycetes subclasses.

Our phylogenetic circumscription of the Acarosporaceae includes Acarospora, Glypholecia, Pleospidium, Polysporina, Sarcogyne, and Thelocarpella, and excludes Biatoridium, Maronea, Sarcosagium, Sporastatia, Strangospora, and Thelocarpus. Our circumscription of the family is most similar to the delimitations given by Eriksson et al. (2004) and Hafellner (1995). However, there are three differences between these classifications and our phylogenetic circumscription. Due to the lack of material, we were not able to verify the placement of Lythoglypha in the Acarosporaceae, as suggested by both Eriksson et al. (2004) and Hafellner (1995). Because the genus Thelocarpella was described in 1999 by Navarro-Rosíes et al. (1999) it could not be considered by Hafellner in 1995, but was accepted as part of the Acarosporaceae by Eriksson et al. (2004) and Kirk et al. (2001). Finally, the genus Pleospidium, which is part of the Acarosporaceae in our study, was placed in the Lecanoraceae by several authors (Eriksson et al., 2004; Hafellner, 1993, 1995; Kirk et al., 2001; Tehler, 1996), or was thought to share some characters with the Candelariaceae (Hertel and Rambold, 1995). Our placement of Pleospidium within the Acarosporaceae confirms Bellemère’s (1994) view that the ontogeny and basic ascal structures of Pleospidium are identical to those of Acarospora and, therefore, Pleospidium should be kept within the Acarosporaceae. It is too early at this point to make any conclusions about relationships among species within the Acarosporaceae. However, we do note that neither Acarospora nor Sarcogyne form monophyletic groups in our analyses. Future studies are needed to establish the delimitation of these two genera.

Maronea forms a well-supported monophyletic clade together with Fuscidea (Figs. 1 and 2), which confirms its placement within the family Fuscidiaceae in accordance with Eriksson et al. (2004), Hafellner (1995), Kirk et al. (2001), Tehler (1996), and Terrón (2000). Sporastatia is definitely not part of the Acarosporaceae. Its placement within the Lecanoromycetidae can be asserted with high confidence, but, without other representatives from the Catillariaceae, we cannot confirm its proposed affiliation to this family (Bellemère, 1994; Eriksson et al., 2004; Hafellner, 1995; Rambold and Triebel, 1992; Tehler, 1996).

The genera Biatoridium, Sarcosagium, Strangospora, and Thelocarpus are excluded from the Acarosporaceae in the present study, albeit with low confidence. It is important to note that even if the exclusion of Biatoridium, Strangospora, and Thelocarpus from the Acarosporaceae is not supported using ML-BP values (≥70% criterion), this exclusion is highly supported by PP ≥ 95% and B-BP ≥ 70% (Fig. 1). The exact placement of Biatoridium within the Lecanorales has not been established (Eriksson et al., 2004; Hafellner, 1994). Our results suggest that this genus is probably not part of the Lecanoromycetes but instead might be closely related to
the Thelocarpaceae. The genus *Thelocarpus* (Thelocarpaceae) seems also not to be a member of the Lecanoromycetes, and its placement in a separate family seems justified. However, placement of the family Thelocarpaceae, probably quite basal within the Pezizomycotina, remains uncertain. At this point the closest relatives to the Thelocarpaceae and *Biatoridium* are the Lichinomycetes, but this inference is supported only by a 100% PP on a short branch. The placement of *Strangospora* also remains unsettled. Because of the lack of support and high phylogenetic uncertainty on the MP tree (Fig. 2), the genus *Strangospora* could still group with *Scoliciosporum* as suggested by Hafellner (1995), but it is clearly not part of the Fusidaceae as Harris et al. (1988) suggested. *Sarcosagium* groups with *Biatoridium* in the 89 SSU + LSU parsimony analyses (76% MP-BP, Fig. 2), but because of a lack of resolution in our phylogenetic reconstruction, we could neither place it in the Thelocarpaceae, as did Eriksson et al. (2004) and Hafellner (1995), nor exclude it from the Aecarosporaceae, where Kirk et al. (2001) and Tehterl (1996) kept it. The grouping of *Biatoridium* with *Sarcosagium* also needs confirmation.

4.4. Newly resolved relationships within the Ascomycota

The monophyly of the Eurotiomycetidae (including the plectomycetes and Chaetothyriales), recovered here with PP = 100%, B-BP = 89%, and ML-BP = 62%, has been reported several times in previous broad phylogenetic studies of the ascomycetes based on nuclear SSU, LSU, and mitochondrial SSU rDNA, as well as on *RPB2*. For example, Geiser and LoBuglio (2001), Inderbitzin et al. (2001), Kauff and Lutzoni (2002, on their ML tree), Liu et al. (1999, on their MP tree), Lumbsch et al. (2001), Miadlikowska and Lutzoni (2004), Platt and Spatafora (2000), Spatafora (1995), and Suh and Blackwell (1999) all found this close relationship between the plectomycetes and Chaetothyriales but without support, whereas, Ekman and Tønsberg (2002), Lücking et al. (2004), Lumbsch et al. (2004, 2002), and Lutzoni et al. (2004, 2001) recovered this relationship with significant PP, and Berbee (1996), Berbee et al. (2000), Gernandt et al. (2001), Ogawa et al. (1997), Okada et al. (1998), and Spatafora et al. (1995) obtained NJ, MP, or ML bootstrap proportions ≥70% for this relationship. Only in a few studies did the Eurotiomycetidae as recognized here not form a monophyletic group. However, the dismemberment of the Eurotiomycetidae was not supported in Kauff and Lutzoni (2002, on their MP tree) and Liu et al. (1999, on their NJ tree) and was supported in Liu and Hall (2004) only with a high PP(98%), while their MP-BP equal 56%. This conflicting result between Liu et al. (2004) and most previous studies is due in part to a very short internode supporting the two competing hypotheses (monophyletic plectomycetes-Chaetothyriales group vs. Chaetothyriales forming a monophyletic group with the rest of the loculoascomycetes). Simulation studies have shown that very short internodes are more likely to support wrong relationships and can receive high posterior probabilities with the current version of MrBayes (Alfaro et al., 2003). When *RPB2* is combined with nuclear SSU and LSU or with nuclear SSU, LSU and mitochondrial SSU, the monophyletic plectomycetes-Chaetothyriales group hypothesis is preferred (with a PP of 100% for the four-gene analysis; Lutzoni et al., 2004). When *RPB2* is analyzed alone, the Chaetothyriales part of the loculoascomycetes hypothesis seems to be preferred (PP = 98%). Because of the very small length of the internodes supporting these two hypotheses, a more extensive taxon sampling and more characters are needed to resolve this issue with high confidence. Finally, our delimitation of the Eurotiomycetidae also comprises the Pyrenulales (∼286 species) and Verrucariales (∼335 species), which include mostly lichen-forming fungi. Liu and Hall (2004) included a member of the Verrucariales (*Dermatocarpon reticulatum*) within the Chaetothyriales, suggesting that the Verrucariales should be subsumed within the Chaetothyriales. To our knowledge, there is not enough evidence at this time or a need to make this major change in the classification of the Ascomycota.

Based on our study, it is not certain whether the subclass Eurotiomycetidae sensu Kauff and Lutzoni (2002) and Taylor et al. (in press) should be kept as part of the Lecanoromycetes, or should form an independent class sister to the rest of the Lecanoromycetes. The Eurotiomycetidae differs notably from the rest of the Lecanoromycetes in terms of several morphological characters: the presence of pseudothecial or cleistotheccal ascomata; hamathecia formed by a combination of periphyses, pseudoparaphyses, short paraphyses (= periphysoids, see Roux and Triebel, 1994; Roux et al., 1995), evanescent paraphyses or lacking one or all of these elements; and bitunicate ascii with forcible discharge or evanescent. It therefore appears that the Eurotiomycetidae would be better placed outside the Lecanoromycetes. Our phylogenetic reconstruction (Fig. 1), as well as that of Kauff and Lutzoni (2002), supports their sister relationship to the Lecanoromycetes (see the other potential Lecanoromycetes node in Fig. 1) and the recognition of this clade at the class level, but the support values were not statistically significant. Lumbsch et al. (2004) and Lutzoni et al. (2004) also rejected the placement of the Eurotiomycetidae within the Lecanoromycetes, this time with highly significant PP.

Because of our limited sampling for the Agyriaceae (i.e., *Placopsis*) and because the monophyly of the family remains questionable, we kept this family within the Ostropales s. l. as suggested by Kauff and Lutzoni (2002). Lumbsch et al. (2001) elevated the Agyriaceae to
the order Agryiales; however, the monophyly of the order was not supported. Both Kauff and Lutzoni (2002) and Miadlikowska and Lutzoni (2004) found the Agryiales to be paraphyletic (without support). Finally, the Agryiales seems to be polyphyletic in a recent study by Lumbsch et al. (2004) including a broad sampling for the Agryiales; a result supported by Lutzoni et al. (2004) with high posterior probabilities. Only Lücking et al. (2004) recovered the Agryiales as monophyletic, but their sampling was limited compared to that of Lumbsch et al. (2004), and the support was based only on Bayesian PP. Both the monophyly and the exact placement of the Agryiales remain to be determined.

With a broader taxon sampling, and by adding the RBP2 gene to the analyses, we showed that the Pertusariales + Icmadophilaceae clade is sister to the Ostropales + Baeomyctales + Hymeneliaceae clade, rather than to the Lecanoromycetidae (Kauff and Lutzoni, 2002; Lutzoni et al., 2001). Lumbsch et al. (2004), as well as Lücking et al. (2004) found the Pertusariales more closely related to the Lecanorales than to the Ostropales s.l., with 100% Bayesian PP and no support, respectively. However, a close relationship between members of the Ostropales s.l. and the Pertusariales was already shown in previous phylogenetic studies, either with significant support (Ekman and Tønsberg, 2002) or without support (Bhattacharya et al., 2000; Lumbsch et al., 2001, 2002; Stenroos and DePriest, 1998; Stenroos et al., 2002; Tehler et al., 2003). As in our study, Miadlikowska and Lutzoni (2004) recovered a sister relationship between the Pertusariales + Icmadophilaceae and Baeomyctales + Ostropales clades (BP = 70%), and formally introduced the subclass Ostropomycetidae to accommodate this relationship. Because the phylogenetic placement of the Pertusariales and Icmadophilaceae within the Ostropomycetidae was strongly supported by our study and by a four-gene phylogenetic analysis in Lutzoni et al. (2004), we propose the establishment of this new subclass within the Lecanoromycetes.

**Subclass.** Ostropomycetidae V. Reeb, Lutzoni and C. Roux subclass. nov.

**Diagnosis.** Fungi non-lichenisati vel cum crustaceo, squamuloso vel filamentoso thallo lichenisati; photobionti chlorococcoid vel trentepohlioidi; ascorma immersa, sessilia vel pedunculata, in forma apotheciorum (cryptolecanorinorum, lecanorinorum, rarius lecideino-immersorum), vel in forma peritheciiorum; 8 vel minus quam 8 ascosporae in ascis, incolores, simplices, transversamente saepitate vel murales; paraphyses s. l. simplices vel plus minusve ramoso-anastomosae; asci unitunicati vel bitunicati sed officio unitunicati, sine tholo vel cum tholo amyloidi aut non, cum vel sine oculari camera.

Non-lichenisi or lichenizati fungi with thallus crustose, squamulose or filamentose; photobionti chlorococcoid or trentepohlioidi; ascomata immersed, sessile or pedunculate in form of apothecia (cryptolecanorine, lecanorine, rarely lecideine-immersed) or in form of perithecia; ascospores eight or fewer per ascus, colorless, simple, transversely septate or muriform; paraphyses s. l. simple or more or less branched-anastomose; ascii unitunicate or bitunicate but functionally ununiticate, lacking tholus and if tholus present amyloid or not, with or without ocular chamber.

**Type order.** Ostropales Nannf.; type family—Stictidaceae Fr. (syn. Ostropaceae Rehm); type genus—*Stictis* Pers.

Members of the Lichinales often have been placed within the Lecanoromycetes (Eriksson et al., 2004), Lecanoromycetidae (Kirk et al., 2001), and Lichinaceae as part of the Lecanorales (Poelt, 1973). Based on morphological characters, Tehler (1996) proposed a basal placement of the Lichinales and Caliciales, sister to the rest of the Pezizomycotina. Recent molecular work has shown that this order is one of the most basal clades within the lichenized ascomycetes, but remains within the Pezizomycotina clade (Kauff and Lutzoni, 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004). Kauff and Lutzoni (2002) elevated the Lichinales, which form an independent lineage, to the level of subclass (Lichinomycetidae). Miadlikowska and Lutzoni (2004), as well as Taylor et al. (in press), referred to the Lichinales as the class Lichinomycetes. The exclusion of the Lichinales from the Lecanoromycetes in our tree (Fig. 1) was well supported by PP, but did not receive B-BP or ML-BP ≥ 70%. However, its closer relationship to the Lecanoromycetes–Eurotiomycetes clade (with high PP in Lutzoni et al., 2004) than to the Sordariomycetes and most Leotiomycetes supports its recognition at the class level. Therefore, we propose here the use of the class Lichinomycetes to encompass all members of the order Lichinales.

**Class.** Lichinomycetes V. Reeb, Lutzoni and C. Roux class. nov.

**Diagnosis.** Thallus crustaceus, squamulosus, foliaceo-umbilicatus vel fruticulosus (humilis statura), generaliter gelatinosus; photobionti cyanobacterii pertinens (praeter raras exceptiones); ascomata initio perithecioidia, sed quae generaliter apothecioidei fluit, immersa vel sessilia, e pycnidii, generantibus hyphis vel thallo formatas; asci generaliter prototunicati, aliqua unitunicati cum rostrali dehiscencia, octosporati vel polysporati (usque ad circa 100 sporas in singulo asco); spora simplices, incolores.

Thallus crustose, squamulose, foliose-umbilicate or fruticose (of small size), generally gelatinous; photobiont cyanobacterial (except in rare cases); ascomata initially perithecioid, generally becoming apothecoid, immersed or sessile, formed from pycnidia, generative hyphae or thallus; asci generally prototunicate, sometimes unitunicate with rostrate dehiscence, octosporous or polysporous (up to 100 spores per ascus); spores simple and colorless.
Type order. Lichinales Hensen and Büdel; type family—Lichinaceae Nyl.; type genus—Lichina C. Agardh.

The delimitation of the Lecanoromycetidae is still not clear and depends, in part, on the resolution and support of basal relationships within the Lecanoromycetes, such as the placement of the Fuscidiaceae–Umbilicariaceae group and the genus *Strangospora* (Fig. 1). Nevertheless, we found that the Fuscidiaceae does not belong in the suborder Teloschistinae as currently reported by Eriksson et al. (2004), or in the order Teloschistales as reported by Kirk et al. (2001) (see Teloschistaceae in Fig. 1). The Fuscidiaceae–Umbilicariaceae group could be recognized as a new order (Umbilicariales) when more members of these two families are included in phylogenetic studies, but should be seen, at the moment, as *incertae sedis* within the Lecanoromycetes.

The differences found between our study and the study by Liu and Hall (2004) probably reside in the fact that they used a single gene and based their conclusions almost exclusively on Bayesian posterior probabilities. We found that our ribosomal gene phylogenies were not in conflict with our *RPB2* phylogeny when we used an NJ-ML 70% bootstrap criterion (see Section 3.2) and that the addition of *RPB2* to ribosomal genes increase both resolution and phylogenetic confidence (see Table 2). We also demonstrated here that silent mutations at the third codon position of *RPB2* provide valuable phylogenetic signal that does not conflict with signal from the first and second codon positions (see Section 3.2). Finally, care must be taken when assessing confidence of phylogenetic relationships using only Bayesian PP as generated by MrBayes v3.0b4 and earlier versions (see Section 4.2; Alfaro et al., 2003; and Douady et al., 2003a). Virtually all conflicts between our results and those of Liu and Hall (2004) vanish when we consider relationships with bootstrap values $\geq 70\%$ from their MP analysis.

4.5. Evolution of polyspory

In the past, all lichen species with polyspored asci including more than 100 spores were automatically placed within the family Acarosporaceae, implying that polyspory with large numbers of spores originated only once during the evolution of lichenized ascomycetes. That hypothesis was questioned, however, as various authors noticed morphological and anatomical inconsistencies among Acarosporaceae species s. l., and began to place members of the Acarosporaceae in different families. Our results reveal that polysporous species do not form a monophyletic group (Fig. 1). Reconstruction of ancestral states for the number of spores per ascus on our 83 SSU + LSU + *RPB2* phylogeny (Fig. 3) shows, depending on the type of reconstruction considered, that polyspory could have originated independently five to six times (reconstruction A), or as little as a single time at the base of the Pezizomycotina (i.e., reconstruction E on node 2 is largely equivocal, but 36% of the trees showed TP > 100 as ancestral state). However, the hypothesis of a single origin of TP > 100, suggesting the occurrence of several reversals to satisfy the species distribution on our phylogenetic tree, is not strongly supported. In the absence of more data, we recognize origins of TP > 100 (marked by black arrows, Fig. 3) to be: (1) at the base of the Acarosporaceae (node 5), with a probable reduction of the number of spores formed within asci for both *Acarospora macrospora* and *Glypholecia scabra* (white diamonds on Fig. 3); (2) on the branch leading to the *Sporastatia* clade (node 16); and (3) on the branch leading to the *Strangospora* clade (node 17). Because of its lack of support, as well as the length of the branches that follow, node 19 was variously equivocal or was assigned state 1, depending on the type of reconstruction. Therefore, polyspory for that group could have originated once at the base of the *Biatoridium–Thelocarpon* clade (black arrow, Fig. 3), or independently on both the *Biatoridium* branch and the *Thelocarpon* branch before node 20 (open arrows, Fig. 3). Finally, node 11 showed either state 0 or equivocal reconstruction, suggesting that polyspory found in *Maronea* more likely originated on the branch leading to that genus.

It is interesting to note that the number of trees having character state 1 at a given node generally increases when *A. macrospora* and *G. scabra* are assigned character state 0 instead of character state 1. This result is somewhat counterintuitive, as we would expect the number of trees with character state 0 at a given node to increase when having more taxa scored as 0 in a data set. However, both *A. macrospora* and *G. scabra* are nested in a highly supported clade that shows character state 1 as the unique ancestral state. This causes a shift in the rate of losses for TP > 100 from 7.2 $\times 10^{-7}$ in reconstruction A to 3.8 in reconstruction E, while values for the rate of gains stay within an average of 0.32 (rates estimated on a single and identical tree across the five reconstructions; results not shown). As the rate of losses increases across reconstructions, TP > 100 is more likely to be gained at the base of the tree, with several subsequent losses, than gained multiple times with only rare losses.

These conclusions on the origin of true polyspory must be drawn with caution, because only true polysporous species with over 100 spores per ascus from lichenized ascomycetes were included in the reconstructions. However, several groups within the non-lichenized ascomycetes also have true polysporous asci with over 100 spores (e.g., *Podospora* and *Diatrypella*). In addition, the distinction between TP > 100 and TP < 100 seems rather arbitrary. One could wonder whether the number of spores per ascus was counted accurately by taxonomists and whether the number of mitoses that occur after meiosis has real systematic value. To confirm this, we would need to incorporate lichenized and non-lichenized species with TP < 100, and assign character states.
appropriately while reconstructing ancestral states. Finally, although Mesquite can take into account phylogenetic reconstruction uncertainties during ancestral state reconstruction (reconstruction over 4000 Bayesian trees), the maximum likelihood approach used in Mesquite seems quite sensitive to character coding and special attention should be given when assigning character states to taxa.

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