Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular Phylogenetics and Evolution 32 (2004) 1036–1060

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

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,[a,*] François Lutzoni,[a] and Claude Roux[b]

[a] *Department of Biology, Duke University, Durham, NC 27708-0338, USA*
[b] *CNRS, UPRES A 6116, Laboratoire de botanique et écologie méditerranéenne, Institut méditerranéen d'écologie et de paléoécologie, Faculté des sciences et techniques de St-Jérôme, case 461, rue Henri Poincaré, F-13397 Marseille cedex 20, France*

Received 15 March 2004; revised 9 April 2004

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).

Keywords: Acarosporomycetidae; Ancestral state reconstruction; Bayesian inference; Detection of topological conflicts; Large-scale Ascomycota phylogeny; Lichinomycetes; Ostropomycetidae; Phylogenetic accuracy

---

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; Lücking et al.,

---

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2004.04.012.

[*] Corresponding author. Fax: 1-919-660-7293.

E-mail address: vreeb@duke.edu (V. Reeb).

1055-7903/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2004.04.012

2004; Lumbsch et al., 2002; Lutzoni et al., 2001; Miadlikowska and Lutzoni, 2004) generally have failed to resolve many of the deep relationships within the Pezizomycotina. Consequently, the establishment of a stable supraordinal classification of the Pezizomycotina remains challenging.

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 (*RPB*2). 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 *RPB*2) of 157 species and a four-locus-based study (SSU nrDNA, LSU nrDNA, mitochondrial small subunit RNA gene, and *RPB*2) 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 *RPB*2 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. placodiiformis*, 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 polyspory 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 (Vězda, 1978), among others (see also Eriksson and Hawksworth, 1996). According to Hafellner (1995) and Ozenda and Clauzade (1970), true polyspory is well known from the lichen order Lecanorales (Lecanoromycetes, Ascomycota) where the Acarosporaceae have been placed. It is therefore possible that true polyspory 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 *Sarcogyne* (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 *Timdalia* are

Table 1
Genera included in the Acarosporaceae according to selected classifications and a new classification proposed here

Taxon	Zahlbruckner, 1907	Magnusson, 1936	Poelt, 1973	Golubkova, 1988	Eriksson and Hawksworth, 1991	Hafellner, 1995	Tehler, 1996	Kirk et al., 2001	Eriksson et al., 2004	This study
Acarospora A. Massal. (1852)	+	+	+	+	+	+	+	+	+	+
Ahlesia Fuckel (1870)	(+)[d]	+	+	.	(+)[d]	.	.	(+)[d]	.	na
Biatorella de Not. (1846)	+	+	+	+	+	–	+	–	–	na
Biatoridium J. Lahm (1860)	(+)[b]	(+)[b]	.	(+)[b]	(+)[b]	.	.	–	–	–
Endocena Cromb. (1876)	–	.	–	.	.	.	–	+	.	na
Eschatogonia Trevis (1853)	–	.	.	.	–	.	–	+	–	na
Glypholecia Nyl. (1853)	+	+	+	+	+	+	+	+	+	+
Lithoglypha Brusse (1988)	.	.	.	.	+	+	+	+	+	na
Maronea A. Massal. (1856)	+	+	+	+	–	–	–	–	–	–
Maronella M. Steiner (1959)	.	.	+	+	(+)[b]	–	.	(+)[b]	.	na
Melanophloea P. James & Vězda (1971)	.	.	.	.	+	–	+	+	–	na
Phacopsis Tul. (1852)	–	.	.	.	–	.	–	+	–	na
Pleopsidium Körber (1855)	(+)[a]	(+)[a]	(+)[a]	(+)[a]	(+)[a]	–	–	–	–	+
Polysporina Vězda (1978)	(+)[c]	(+)[c]	(+)[c]	+	+	+	+	+	+	+
Sarcogyne Flot. (1851)	(+)[b]	+	+	+	+	+	+	+	+	+
Sarcosagium A. Massal. (1856)	(+)[b]	(+)[b]	.	–	+	–	+	+	–	?
Sporastatia A. Massal. (1854)	(+)[b]	+	+	+	+	–	–	+	–	–
Strangospora Körber (1860)	(+)[b]	(+)[b]	.	+	+	–	–	+	–	–
Thelocarpella Nav.-Ros. & Cl. Roux (1999)	.	.	.	.	.	.	.	+	+	+
Thelocarpon Nyl. (1853)	+	–	+	–	+	–	+	+	–	–
Timdalia Hafellner (2001)	.	(+)[a]	(+)[a]	(+)[a]	(+)[a]	(+)[a]	(+)[a]	(+)[a]	–	na

When possible, synonymy of taxa was taken into account for each classification. "+", taxa present in a given classification. "(+)", 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. "–", taxa not part of the Acarosporaceae. ".", 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 *Biatorella*.
[c] = included in *Sarcogyne*.
[d] = included in *Thelocarpon*.

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 asci have never been observed for these genera. Recent studies have placed *Endocena* in the Icmadophilaceae (Stenroos et al., 2002) and *Phacopsis* in the Parmeliaceae (Peršoh 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 asci. However, these two families have several putatively ancestral features (e.g., a crustose saxicolous thallus, a cryptolecanorine 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 *RPB*2) 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*, *Melanophloea*, and *Timdalia*, 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 infering 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, Icmadophilaceae, 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 *RPB*2 nDNA. Combinations of various primers were used to amplify the SSU (nssu97a, nssu97b, nssu131, NS22, nssu1088), LSU (LR0R, LIC15R, LIC24R, LIC2044, and LR7), and *RPB*2 (f*RPB*2-5F, f*RPB*2-7cF, f*RPB*2-7cR, f*RPB*2-11aR, *RPB*2-608F, *RPB*2-3053R, and *RPB*2-3053bR) 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 *RPB*2 primers, 1 μl of 10 mg/ml bovin serum albumin (New England Biolabs #B9001S), 1 μl of 25 mg/ml $MgCl_2$, 17 μl or 14 μl of sterile double-distilled water for the SSU/LSU or *RPB*2 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 (GELase Agarose Gel-Digesting Preparation, Epicenter #G31100). Sequencing reactions were performed in a 10 μl final volume: 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 $MgCl_2$, 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'-GGTAAGC AGAACTGGCG-3']), and *RPB*2 (*RPB*2-908F, *RPB*2-1014R, *RPB*2-1554F, *RPB*2-1554R, *RPB*2-2473F, *RPB*2-2488F, *RPB*2-2492R, and *RPB*2-2568R) 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 *RPB*2 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 *RPB*2, 83 SSU + LSU + *RPB*2, 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* (Saccharomycetes; GenBank Accession No. U53879) provided by Cannone et al. (2002). The protein-coding gene *RPB*2 was aligned in MacClade using the option "nucleotides with amino acid colors" to facilitate manual alignment. For *RPB*2, 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 $\geqslant 70\%$ or PP $\geqslant 95\%$. 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 *RPB*2 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 + *RPB*2 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 + *RPB*2 data set were subjected to five step matrices, including one for each of the ribosomal genes and one for each of the *RPB*2 codon positions. Frequency of changes were calculated independently for SSU and LSU. However, frequency of changes for each of the *RPB*2 step matrices (*RPB*2-1st, -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, *RPB*2-1st, -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 *RPB*2 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 *RPB*2 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 + *RPB*2 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 + *RPB*2 and 89 SSU + LSU

Table 2
Summary of node support comparisons among different analyses of the 83 SSU + LSU + *RPB*2 and 89 SSU + LSU data sets

Analysis type	83 SSU + LSU + *RPB*2 data set								89 SSU + LSU data set				
	MP-BP	MP + I-BP	MP + I + A-BP	MP + I + A + STprop-BP	**Bayesian 5mod-PP**	Bayesian 1mod-PP	Bootstrapped Bayesian 5mod-B-BP	ML 1mod-BP	MP-BP	MP + I-BP	MP + I + A-BP	MP + I + A + STprop-BP	Bayesian 2mod-PP
No. of replicates or sampled trees	512	512	512	512	**40,000**	40,000	100	100	122	248	505	500	40, 000
No. of internodes recovered	59	59	60	59	**79**	79	69	62	43	51	52	52	73
Total no. of resolvedinternodes in common with the reference	51	51	50	57	–	72	79	76	51	53	55	51	50
Sum of all inter nodes support values	4517	4561	4559	4990	**7693**	7033	6651	6124	3784	4059	4069	4029	4594
No. of significantly supported internodes	43	43	45	46	**68**	63	59	54	33	33	36	37	38
No. of significantly supported internodes in common with the reference	43	43	45	46	–	63	59	54	33	33	36	37	37
No. of significantly supported internodes lost	25	25	23	22	–	5	9	14	35	35	32	31	31
No. of significantly supported internodes gained	0	0	0	0	–	0	0	0	0	0	0	0	1

The five-model Bayesian analysis of the 83 SSU + LSU + *RPB*2 data set (in bold) was used as a reference for all other analyses. Nodes were considered significant if support values were ⩾70% for MP, ML, and Bayesian bootstrap analyses, and ⩾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 + *RPB*2 data set was assumed to have five distinct partitions (SSU, LSU, *RPB*2-1st, -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 were 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 gamma categories 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 bootstrapped 83 SSU + LSU + *RPB*2 data sets. These bootstrapped data sets were assembled by generating 100 bootstrap pseudo-replicates for each of the five partitions (SSU, LSU, *RPB*2-1st, -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+*RPB*2 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 ($r[A<->C]=2.271974$, $r[A<->G]=6.398565$, $r[A<->T]=1.631732$, $r[C<->G]=1.119333$, $r[C<->T]=8.226414$, $r[G<->T]=1.000000$, $pi[A]=0.228385$, $pi[C]=0.261017$, $pi[G]=0.266777$, $pi[T]=0.243821$, $\alpha=0.316064$, $pinvar = 0.300560$). 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 auct.* from other true polyspored 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 + *RPB*2 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 + *RPB*2 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 macrospora* and *Glyphlolecia 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 bombacina*, has basidiospores instead of ascospores and, therefore, it could be argued that basidiospores are not homologous to ascospores. Consequently, *Athelia*

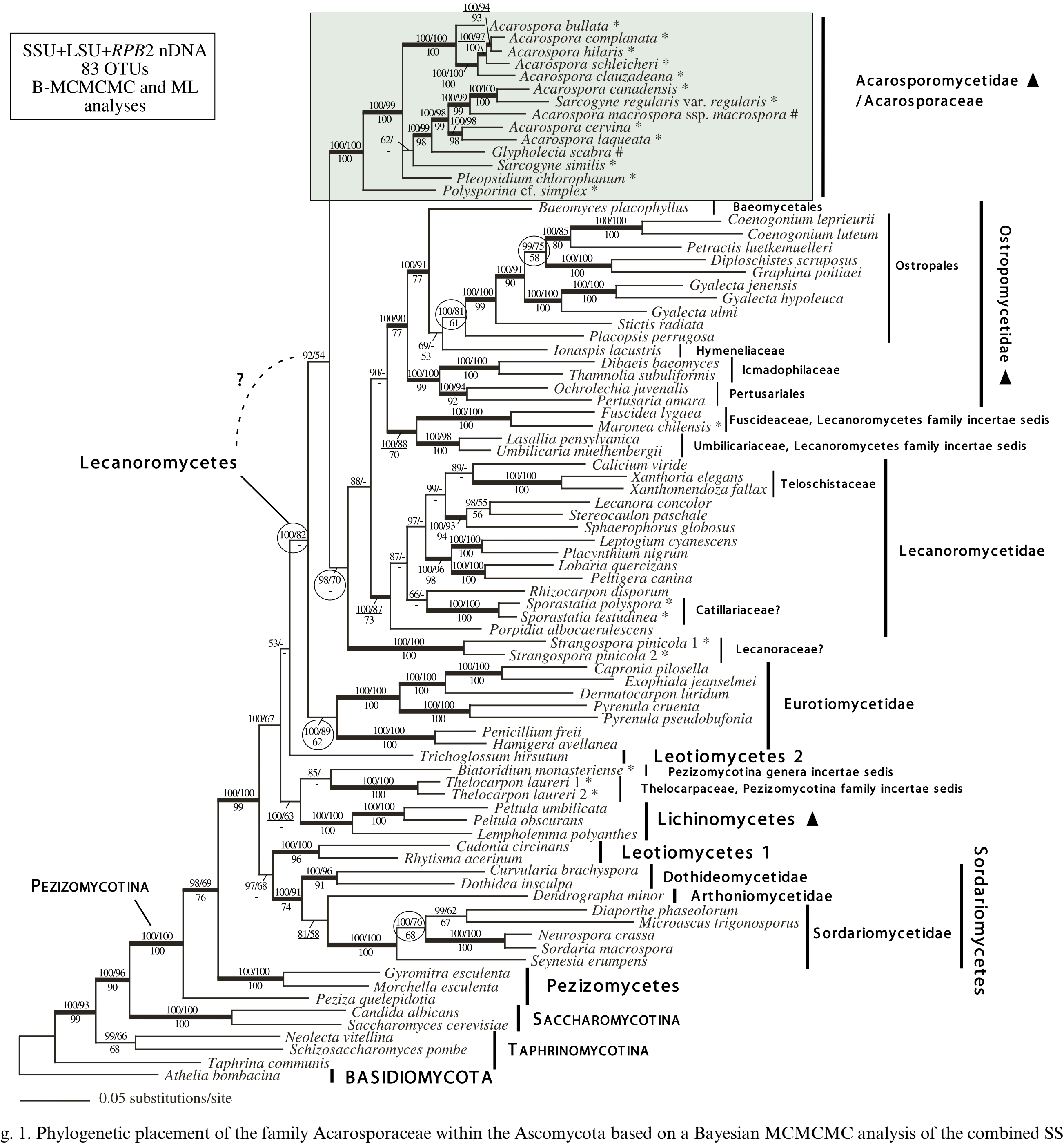

Fig. 1. Phylogenetic placement of the family Acarosporaceae within the Ascomycota based on a Bayesian MCMCMC analysis of the combined SSU nrDNA, LSU nrDNA, and *RPB*2 nDNA data set for 82 species of the Ascomycota, including 14 species from the Acarosporomycetidae/Acarosporaceae, and one basidiomycete species used as outgroup (i.e., 83 SSU + LSU + *RPB*2). The phylogram represents the majority rule consensus tree of 40,000 post-burnin trees sampled by the B-MCMCMC 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 ⩾50% above internodes that are before the backslash are B-MCMCMC 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).

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 + *RPB*2 data set, SSU and LSU together contributed to 77.9% of the total alignment length; nevertheless, the *RPB*2 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, *RPB*2 has only small spliceosomal introns at six splicing sites. Most of the variation in the *RPB*2 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 3
Summary of data sets used for MP, Bayesian, and ML analyses

	83 SSU	83 LSU	83 *RPB*2	83 SSU + LSU + *RPB*2: Bayesian and ML analyses	83 SSU + LSU + *RPB*2: MP analyses	89 SSU	89 LSU	89 SSU + LSU: Bayesian and ML analyses	89 SSU + LSU: MP analyses
No. of sites in alignment	5043	3869	2526	11,438	11,438	5250	3873	9123	9123
No. of excluded sites[a]	4061	2885	750	7696	7696	4268	2889	7157	7157
No. of amb. aligned regions (no. of sites)	22 (179)	23 (341)	10 (414)	55 (934)	55 (934)	22 (179)	23 (345)	45 (524)	45 (524)
No. amb. re-coded (INAASE, arc)	22 (19, 3)	23 (13, 10)	9 (4, 9)	0	54 (36, 22)	22 (19, 3)	23 (13, 10)	0	45 (32, 13)
No. of regions with introns (no. of sites)	15 (3882)	16 (2544)	6 (336)	37 (6762)	37 (6762)	16 (4089)	16 (2544)	32 (6633)	32 (6633)
No. introns re-coded (INAASE, arc)	0	0	1 (0, 1)	0	1 (0, 1)	0	0	0	0
Total no. of sites analyzed	982	984	1776	3742	4216	982	984	1966	2297
No. of constant sites	586	543	606	1735	1752	584	543	1127	1112
No. of variable sites	396	441	1170	2007	2464	398	441	839	1185
No. of parsimony-informative sites	242	331	1075	1648	2105	245	331	576	918

[a] 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 settings 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 estimates 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 distance) bootstrap approach. This conservative approach seems to generate fewer false positives when conducting large-scale phylogenetic studies (MP or ML bootstrap analyses being computationally too time consuming for large-scale phylogenies). Using the reciprocal 70% BP criterion, we detected a lower number of potential conflicts compared to the Bayesian analysis, 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 supported 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 *RPB*2 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 topologies. 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, therefore, 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 *RPB*2-1st, -2nd, and -3rd codon positions. Therefore, we concluded that saturation 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 + *RPB*2 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 internodes 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 bootstrapped 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). However, 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 addition 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 internodes 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 phylogeny (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 advantageous, but did prove itself in MP analyses of the 83 SSU + LSU + *RPB*2 data set.

The Bayesian five-model consensus tree for the 83 SSU + LSU + *RPB*2 data set revealed 68 significantly 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 between the two topologies. The Bayesian two-model analysis (Bayesian 2mod) on the 89 SSU + LSU data set generated two additional significantly supported internodes compared to the MP + I + A analysis; however, the total number of resolved internodes in common with the reference tree decreased by five (50 vs. 55, Table 2). In addition, this Bayesian consensus tree showed some surprising relationships, such as the inclusion of two members of the Eurotiomycetidae within the Acarosporomycetidae clade (PP = 97%), which was not recovered by the 89-taxon MP + I + A analysis, the 83-taxon Bayesian five-model consensus tree, or any other phylogenetic studies of the Ascomycota. Consequently, we have considered only the 83 SSU + LSU + *RPB*2 Bayesian five-model analysis and the 89 SSU + LSU MP + I + A analysis for all following discussions, as these analyses gave the highest level of resolution when implemented on their respective original data sets.

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-MCMCMC 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 $\geqslant$ 95%), 59 had B-BP values $\geqslant$ 70%, and 54 had ML-BP values $\geqslant$ 70% (Table 2). All branches supported with ML-BP $\geqslant$ 70% were also supported by PP $\geqslant$ 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 $\geqslant$ 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 $\geqslant$ 95%, BP $\geqslant$ 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 Lecanoromycetes (100% PP and 82% B-BP); and finally the Acarosporomycetidae 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 + *RPB*2 tree (Fig. 1), the Pezizomycetes form a well-supported paraphyletic group (98% PP, 76% ML-BP), with *Peziza quelepidotia* part of a distinct lineage, and *Gyromitra esculenta* and *Morchella esculenta* sister to the rest of the Pezizomycotina. However, this paraphyletic grouping could be due to long-branch attraction (Felsenstein, 1978) associated with a poor sampling of the Pezizomycetes. A phylogenetic analysis of a combined nuclear SSU + LSU data set of 558 species representing all traditionally recognized fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota) and the Glomeromycota, and which included 21 taxa classified within the Pezizomycetes, 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, Eurotiales, 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 Fuscideaceae (100% PP, 88% B-BP, and 70% ML-BP, Fig. 1). The Pertusariales–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 nom. nov. with high significant support (100% PP, 90% B-BP, and 77% ML-BP).

The Acarosporomycetidae subclass. nov. (Acarosporaceae Zahlbr. in Eriksson et al., 2004), including *Acarospora*, *Glypholecia*, *Pleopsidium*, *Polysporina*, and *Sarcogyne*, forms a well-supported monophyletic group with 100% PP, B-BP, and ML-BP. Two genera can be excluded from the Acarosporomycetidae with high confidence (Fig. 1, Table 1): *Maronea* (shown here to be part of the Fuscideaceae; 100% PP, B-BP, and ML-BP); and *Sporastatia* (included in the Lecanoromycetidae; 100% PP, 87% B-BP, and 73% ML-BP). The exclusion of *Strangospora* from the Acarosporomycetidae is supported by 98% PP and 70% B-BP. This genus appears to form an independent lineage sister to the Ostropomycetidae–Lecanoromycetidaes s. l. clade, but the relationship lacks support. The phylogenetic placement of *Biatoridium* and *Thelocarpon* remains uncertain. However, they are very likely to be excluded from the Lecanoromycetes (100% PP and 82% B-BP) and seem to form a monophyletic group sister to the Lichinomycetes (100% PP and 63% B-BP). Based on the SSU + LSU + *RPB*2 data, the Acarosporomycetidae is sister to the Ostropomycetidae–Lecanoromycetidae group, with the Eurotiomycetidae basal within the Lecanoromycetes. However, while the Ostropomycetidae–Lecanoromycetidae forms a relatively well-supported clade (98% PP and 70% B-BP), relationships with the Acarosporomycetidae and Eurotiomycetidae remain unsupported.

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 *Diaporthe* (Sordariomycetidae), none of the discrepancies between the two topologies were supported by MP-BP $\geqslant 70\%$ 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 Fuscideaceae as revealed by the Bayesian analysis of the 83 SSU + LSU + *RPB*2 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

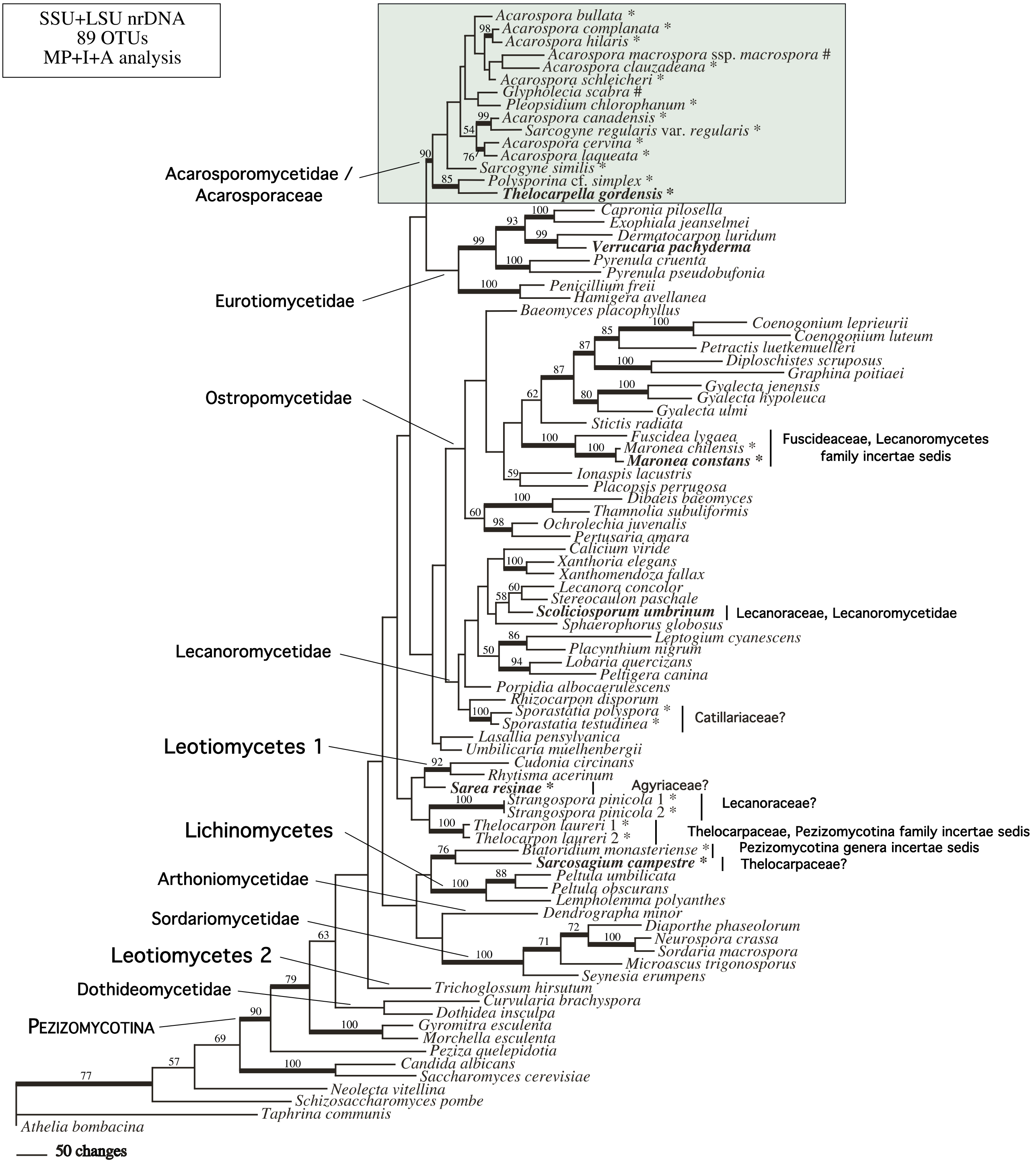

Fig. 2. Phylogenetic circumscription of the Acarosporaceae, and placement of *Sarcosagium*, *Sarea*, *Scoliciosporum*, *Thelocarpella*, and *Verrucaria* within the Pezizomycotina (euascomycetes) based on the combined SSU and LSU nrDNA data set for 88 species of the Ascomycota, including 15 species from 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 ⩾ 50%. If the parsimony bootstrap support was ⩾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.

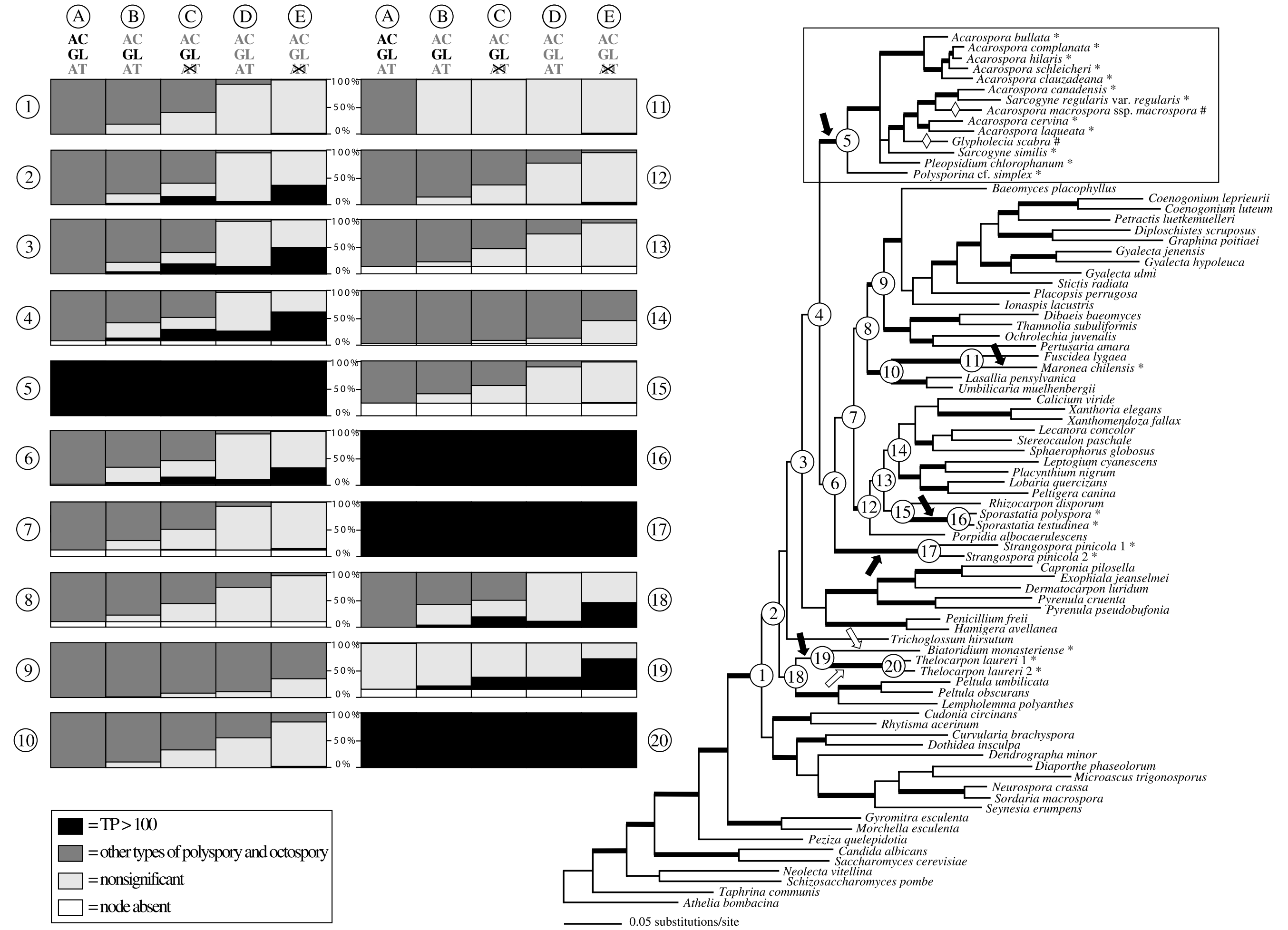

Fig. 3. Evolution of true polyspory with over 100 spores per ascus, reconstructed on 20 nodes for over 4000 Bayesian trees randomly sampled from the pool of 40,000 trees obtained during the Bayesian analysis of the 83 SSU + LSU + *RPB*2 data set (Fig. 1). Five types of reconstruction (A–E) were performed with different character state assignments for *Acarospora macrospora*, *Glypholecia scabra*, and *Athelia bombacina*. A maximum likelihood optimality criterion (Pagel, 1999) and an asymmetric 2-parameter model for rate of gains and losses were applied as implemented in Mesquite 1.0 (Maddison and Maddison, 2003). Taxon names followed by an asterisk correspond to true polyspored species with over 100 spores per ascus and were coded as "1" in the different reconstructions. Taxon names followed by a pound sign are members of the Acarosporaceae s. str. with fewer than 100 spores per ascus, and were coded either 0 or 1. All other taxa were coded 0, except for the outgroup *Athelia bombacina*, which was coded either as 0 (reconstructions A, B, and D) or removed from the analyses (reconstructions C and E). Numbers within the circles are node numbers. Closed arrows indicate internodes where true polyspory may have originated. Open arrows represent alternative hypotheses for the origin of polyspory based on results obtained for reconstruction of ancestral state at node 19. White diamonds indicate potential reduction of the number of spores per ascus. Internodes with PP ⩾ 95% and ML-BP ⩾ 70% are represented by thicker lines. Stacked columns on the left of the figure show, for each node investigated and type of reconstruction performed, the percentage of trees (0–100 scale) supporting a given ancestral state. "AC", *A. macrospora*; "GL", *G. scabra*; and "AT", *Athelium bombacina*. Abbreviations for these three names appear either in black when coded as 1 (TP > 100), in grey when coded as 0 (TP < 100, non-true polyspory and octospory) or crossed when removed from the analyses.

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-taxa 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 + I + 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 *RPB*2 was included in the analyses. *RPB*2 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 + I + 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 *RPB*2 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 + *RPB*2 data set gave greater support than when using a single model for all three genes, likely reflecting differences in model of evolution between *RPB*2 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 INAASE (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 nonparametric 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 + *RPB*2 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 $\geqslant 95\%$ than by ML-BP $\geqslant 70\%$, 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 $\geqslant 95\%$ 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., $\geqslant 70\%$), 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 polytomic trees (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 $\geqslant 95\%$, and the lower, accuracy of ML-BP, but with lower numbers of wrong clades with support values $\geqslant 70\%$.

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 Acarosporineae 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 Acarosporineae 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 chlorococcoide; ascomata immersa vel sessilia, in forma apotheciorum (cryptolecanorinorum, lecanorinorum vel lecideinorum, rarius biatorinorum vel pseudolecanorinorum), raro in forma peritheciorum; ascosporae generaliter plus quam centum in ascis, simplices et incolores, halo expertes; paraphyses s. l. mediocriter vel infirme ramoso-anastomosae; asci bitunicati, officio unitunicati, cum tholo non-amyloidi vel satis infirme amyloidi, 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; asci bitunicate, functionally unitunicate, non-amyloid or slightly amyloid tholus, presence of ocular chamber.

Circumscription. See Fig. 1.

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

Previous phylogenies failed to reveal relationships among the four subphyla of the Lecanoromycetes (Ekman and Tønsberg, 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*, *Pleopsidium*, *Polysporina*, *Sarcogyne*, and *Thelocarpella*, and excludes *Biatoridium*, *Maronea*, *Sarcosagium*, *Sporastatia*, *Strangospora*, and *Thelocarpon*. 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-Rosinés 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 *Pleopsidium*, 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 *Pleopsidium* are identical to those of *Acarospora* and, therefore, *Pleopsidium* 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 *Thelocarpon* 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 *Thelocarpon* from the Acarosporaceae is not supported using ML-BP values ($\geqslant$70% criterion), this exclusion is highly supported by PP $\geqslant$ 95% and B-BP $\geqslant$ 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 *Thelocarpon* (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 Fuscideaceae 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 Acarosporaceae, where Kirk et al. (2001) and Tehler (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 *RPB*2. 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 *RPB*2 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 *RPB*2 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 cleistothecial 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 asci 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 Agyriales; however, the monophyly of the order was not supported. Both Kauff and Lutzoni (2002) and Miadlikowska and Lutzoni (2004) found the Agyriaceae to be paraphyletic (without support). Finally, the Agyriales seems to be polyphyletic in a recent study by Lumbsch et al. (2004) including a broad sampling for the Agyriales; a result supported by Lutzoni et al. (2004) with high posterior probabilities. Only Lücking et al. (2004) recovered the Agyriales 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 Agyriaceae remain to be determined.

With a broader taxon sampling, and by adding the *RBP*2 gene to the analyses, we showed that the Pertusariales + Icmadophilaceae clade is sister to the Ostropales + Baeomycetales + 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 Baeomycetales + Ostropales clades (BP = 70%), and informally introduced the subclass Ostropomycetidae to accommodate this relationship. Because the phylogenetic placement of the Pertusariales and Icmadophylaceae 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; photosymbiota chlorococcoide vel trentepohlioide; ascomata immersa, sessilia vel pedunculata, in forma apotheciorum (cryptolecanorinorum, lecanorinorum, rarius lecideino-immersorum), vel in forma peritheciorum; 8 vel minus quam 8 ascosporae in ascis, incolores, simplices, transverse saeptatae 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-lichenized or lichenized fungi with thallus crustose, squamulose or filamentose; photobiont chlorococcoid or trentepohlioid; 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; asci unitunicate or bitunicate but functionally unitunicate, 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 Lichinineae 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 $\geqslant 70\%$. 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 (humili statura), generaliter gelatinosus; photosymbiota cyanobacteriis pertinens (praeter raras exceptiones); ascomata initio perithecioidia, sed quae generaliter apothecioidia fiunt, immersa vel sessilia, e pycnidiis, generantibus hyphis vel thallo formata; asci generaliter prototunicati, aliquando unitunicati cum rostrali dehiscentia, octosporati vel polysporati (usque ad circa 100 sporas in singulo asco); sporae 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 Fuscideaceae–Umbilicariaceae group and the genus *Strangospora* (Fig. 1). Nevertheless, we found that the Fuscidiaceae does not belong in the suborder Teloschistineae 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 Fuscideaceae–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 *RPB*2 phylogeny when we used an NJ-ML 70% bootstrap criterion (see Section 3.2) and that the addition of *RPB*2 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 *RPB*2 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 relationship 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 $\geqslant$ 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 + *RPB*2 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 7.2×10^{-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 polyspored 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 polyspored 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.

Acknowledgments

We thank Katie Glew, Sabine Huhndorf, Frank Kauff, Martin Kukwa, Elisabeth Lay, Thierry Ménard, Jolanta Miadlikowska, Kerry O'Donnell, Iris Pereira, Christoph Scheidegger, Keith Seifert, Felix Schumm, and Becky Yahr, as well as the curators of the Herbaria of ASU, CANL, COLO, H, LOD-L, O, and TBS, for providing dry material or cultures used in this study. We are grateful to Jamie Platt and Ulrik Søchting for providing DNA; Stefan Zoller for help in using the IBM RS/6000 SP Unix systems at the North Carolina Supercomputing Center; Frank Kauff for writing computer programs necessary for this study; Jolanta Miadlikowska for her support and useful discussions; Elizabeth Arnold and Mary Berbee for comments on the manuscript; Molly McMullen for editing; and Michelle Roux for Latin translation of the diagnoses. This research was performed at both the Field Museum's Pritzker Laboratory of Molecular Systematics and Evolution (operated with support from an endowment from the Pritzker Foundation), and Duke University. We gratefully acknowledge support from four grants from the National Science Foundation, USA (Systematic Biology DEB-9615542 and CAREER DEB-0133891 to FL; Assembling the Tree of Life [ATOL] DEB-0228668 to FL and R. Vilgalys; and Doctoral Dissertation Improvement Grant DEB-0105194 to FL and VR), a Karling Graduate Student Research Award from the Botanical Society of America to VR, an American Society of Plant Taxonomists Graduate Research Award to VR, and a Graduate Fellowship from the A. W. Mellon Foundation, Plant Systematics Training Program, Duke University to VR.

References

Alexopoulos, C.J., Mims, C.W., Blackwell, M., 1996. Introductory Mycology. Wiley, New York.

Alfaro, M.E., Zoller, S., Lutzoni, F., 2003. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Mol. Biol. Evol. 20 (2), 255–266.

Berbee, M.L., 1996. Loculoascomycete origins and evolution of filamentous Ascomycete morphology based on 18S rRNA gene sequence data. Mol. Biol. Evol. 13 (3), 462–470.

Berbee, M.L., Carmean, D.A., Winka, K., 2000. Ribosomal DNA and resolution of branching order among the Ascomycota: how many nucleotides are enough? Mol. Phylogenet. Evol. 17 (3), 337–344.

Bellemère, A., 1994. Documents et commentaires sur l'ultrastructure des asques polysporés des *Acarospora*, de quelques genres de la famille des Acarosporaceae et de genres similaires. Bull. Soc. Linn. Provence 45, 355–388.

Bhattacharya, D., Friedl, T., Helms, G., 2002. Vertical evolution and intragenic spread of lichen-fungal group I introns. J. Mol. Evol. 55, 74–84.

Bhattacharya, D., Lutzoni, F., Reeb, V., Simon, D., Nason, J., Fernandez, F., 2000. Widespread occurrence of spliceosomal introns in the rDNA genes of Ascomycetes. Mol. Biol. Evol. 17 (12), 1971–1984.

Buckley, T.R., 2002. Model misspecification and probabilistic tests of topology: evidence from empirical data sets. Syst. Biol. 51 (3), 509–523.

Buckley, T.R., Arensburger, P., Simon, C., Chambers, G.K., 2002. Combined data, Bayesian phylogenetics, and the origin of the New Zealand cicada genera. Syst. Biol. 51 (1), 4–18.

Bull, J.J., Huelsenbeck, J.P., Cunningham, C.W., Swofford, D.L., Waddell, P.J., 1993. Partitioning and combining data in phylogenetic analysis. Syst. Biol. 42 (3), 384–397.

Cannone, J.J., Subramanian, S., Schnare, M.N., Collett, J.R., D'Souza, L.M., Du, Y., Feng, B., Lin, N., Madabusi, L.V., Muller, K.M., Pande, N., Shang, Z., Yu, N., Gutell, R.R., 2002. The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BioMed Central Bioinformat. 3, 2, Correction: BioMed Central Bioinformatics 3, 15. Database available from http://www.rna.icmb.utexas.edu/.

Craven, K.D., Hsiau, P.T.W., Leuchtmann, A., Hollin, W., Schardl, C.L., 2001. Multigene phylogeny of *Epichloe* species, fungal symbionts of grasses. Ann. Missouri Bot. Gard. 88 (1), 14–34.

Cummings, M.P., Handley, S.A., Myers, D.S., Reed, D.L., Rokas, A., Winka, K., 2003. Comparing bootstrap and posterior probability values in the four-taxon case. Syst. Biol. 52 (4), 477–487.

Dodge, C.W., 1973. Lichen Flora of the Antarctic Continent and Adjacent Islands. Phoenix, Canaan, New Hampshire.

Douady, C.J., Delsuc, F., Boucher, Y., Doolittle, W.F., Douzery, E.J.P., 2003a. Comparison of the Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol. Biol. Evol. 20 (2), 248–254.

Douady, C.J., Dosay, M., Shivji, M.S., Stanhope, M.J., 2003b. Molecular phylogenetic evidence refuting the hypothesis of Batoidea (rays and skates) as derived sharks. Mol. Phylogenet. Evol. 26, 215–221.

Efron, B., Halloran, E., Holmes, S., 1996. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. USA 93, 13429–13434.

Ekman, S., 1996. The corticolous and lignicolous species of *Bacidia* and *Bacidina* in North America. Opera Bot. 127, 1–148.

Ekman, S., Tønsberg, T., 2002. Most species of *Lepraria* and *Leproloma* form a monophyletic group closely related to *Stereocaulon*. Mycol. Res. 106, 1262–1276.

Elenkin, A.A., 1911. Flora lishajnikov Srednej Rossii. Part 3–4. Juriev.

Eriksson, O.E., 1999. Outline of Ascomycota—1999. Myconet 3, 1–88.

Eriksson, O.E., Baral, H.-O., Currah, R.S., Hansen, K., Kurtzman, C.P., Rambold, G., Laessøe, T., 2001. Outline of Ascomycota—2001. Myconet 7, 1–88.

Eriksson, O.E., Baral, H.-O., Currah, R.S., Hansen, K., Kurtzman, C.P., Rambold, G., Laessøe, T., 2004. Outline of Ascomycota—2004. Myconet 10, 1–99.

Eriksson, O.E., Hawksworth, D.L., 1991. Outline of the Ascomycetes—1990. Syst. Asc. 9 (1–2), 39–271.

Eriksson, O.E., Hawksworth, D.L., 1996. Notes on Ascomycete systematics—Nos 2024–2139. Syst. Asc. 14 (2), 101–133.

Erixon, P., Svennblad, B., Britton, T., Oxelman, B., 2003. Reliability of Bayesian posterior probabilities and bootstrap frequencies in phylogenetics. Syst. Biol. 52 (2), 665–673.

Felsenstein, J., 1978. Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. 27, 401–410.

Felsenstein, J., 1981. A likelihood approach to character weighting and what it tells us about parsimony and compatibility. Bio. J. Linn. Soc. 16, 183–196.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4), 783–791.

Felsenstein, J., 2002. PHYLIP (Phylogeny Inference Package) version 3.6a3. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle. Available from http://evolution.genetics.washington.edu/phylip/phylip36.html.

Geiser, D.M., Frisvad, J.C., Taylor, J.W., 1998. Evolutionary relationships in *Aspergillus* section *Fumigati* inferred from partial beta-tubulin and hydrophobin DNA sequences. Mycologia 90 (5), 831–845.

Geiser, D.M., LoBuglio, K.F., 2001. The monophyletic Plectomycetes: Ascophaerales, Onygenales, Eurotiales. In: McLaughlin, McLaughlin, Lemke, (Eds.), Systematics and Evolution. The Mycota VII Part A. Springer-Verlag, Berlin Heidelberg, pp. 202–219.

Gernandt, D.S., Platt, J.L., Stone, J.K., Spatafora, J.W., Holst-Jensen, A., Hamelin, R.C., Kohn, L.M., 2001. Phylogenetics of Helotiales and Rhytismatales based on partial small subunit nuclear ribosomal DNA sequences. Mycologia 93 (5), 915–933.

Golubkova, N.S., 1988. Lishajniki semejstva Acarosporaceae Zahlbr. v SSSR, Nauka, Leningrad.

Grantham, R., 1974. Amino acid difference formula to help explain protein evolution. Science 185, 862–864.

Hackett, S.J., 1996. Molecular phylogenetics and biogeography of Tanagers in the genus *Ramphocelus* (Aves). Mol. Phylogenet. Evol. 5 (2), 368–382.

Hafellner, J., 1993. *Acarospora* und *Pleopsidium*—zwei lichenisierte Ascomycetengattungen (Lecanorales) mit zahlreichen Konvergenzen. Nova Hedwigia 56 (3–4), 281–305.

Hafellner, J., 1994. On *Biatoridium*, a resurrected genus of lichenized fungi (Ascomycotina, Lecanorales). Acta Bot. Fennica 150, 39–46.

Hafellner, J., 1995. Towards a better circumscription of the Acarosporaceae (lichenized Ascomycotina, Lecanorales). Crypt. Bot. 5, 99–104.

Harris, R.C., Buck, W.R., Nelson, J., Selva, S.B., Guccion, J.G., Schmitt, C., 1988. Lichens of southern Maine collected on the 1987 Andrews Foray. Evansia 5 (2), 26–32.

Hawksworth, D.L., Kirk, P.M., Sutton, B.C., Pegler, D.N., 1995. Ainsworth & Bisby's Dictionary of the Fungi, eighth ed. CAB International, Wallingford.

Hertel, H., Rambold, G., 1988. *Lecidea mosigii* (Koerb.) Anzi—eine Art der Gattung *Orphniospora* Koerb. (Fuscideaceae, Teloschistales). Mitt. Bot. Staatssamml. München 27, 111–123.

Hertel, H., Rambold, G., 1995. On the genus *Adelolecia* (lichenized Ascomycotina, Lecanorales). In: Knoph, J.-G., Schrüfer, K., Sipman, H.J.M. (Eds.), Studies in Lichenology with Emphasis on Chemotaxonomy, Geography and Phytochemistry. Festschrift Ch. Leuckert. Bibl. Lichenol. 57, J. Cramer, Berlin-Stuttgart, pp. 211–230.

Hillis, D.M., Bull, J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42 (2), 182–192.

Huelsenbeck, J.P., Larget, B., Miller, R., Ronquist, F., 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol. 51 (5), 673–688.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755, Program Available from http://morphbank.ebc.uu.se/mrbayes3/info.php.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Inderbitzin, P., Landvik, S., Abdel-Wahab, M.A., Berbee, M.L., 2001. Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. Am. J. Bot. 88 (1), 52–61.

Kauff, F., Lutzoni, F., 2002. Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships based on nuclear ribosomal RNA small and large subunits. Mol. Phylogenet. Evol. 25, 138–156.

Kirk, P.M., Cannon, P.F., David, J.C., Stalpers, J.A., 2001. Ainsworth & Bisby's Dictionary of the Fungi, ninth ed. CAB International, Wallingford.

Kjer, K.M., 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. J. Mol. Evol. 4, 314–330.

Landvik, S., Eriksson, O.E., Berbee, M.L., 2001. *Neolecta*—a fungal dinosaur? Evidence from beta-tubulin amino acid sequences. Mycologia 93 (6), 1151–1163.

Larget, B., Simon, D., 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Mol. Biol. Evol. 16, 750–759.

Leaché, A.D., Reeder, T.W., 2002. Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): a comparison of parsimony, likelihood, and Bayesian approaches. Syst. Biol. 51 (1), 44–68.

Lewis, P., Holder, M.T., 2003. Examining the effects of branch length priors on clade support. Phylogenetic Methods/Theory I, Session 7e. Evolution meeting, California State University, Chico, June 20–24.

Li, S., 1996. Phylogenetic Tree Construction Using Markov Chain Monte Carlo. Ph.D. dissertation, Ohio State University, Columbus, USA.

Liu, Y.J., Hall, B.D., 2004. Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny. Proc. Natl. Acad. Sci. USA 101 (13), 4507–4512.

Liu, Y.J., Whelen, S., Hall, B.D., 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol. Biol. Evol. 16 (12), 1799–1808.

Lücking, R., Stuart, B.L., Lumbsch, H.T., 2004. Phylogenetic relationships of Gomphillaceae and Asterothyriaceae: evidence from a combined Bayesian analysis of nuclear and mitochondrial sequences. Mycologia 96 (2), 283–294.

Lumbsch, H.T., Schmitt, I., Döring, H., Wedin, M., 2001. Molecular systematics supports the recognition of an additional order of Ascomycota: the Agyriales. Mycol. Res. 105 (1), 16–23.

Lumbsch, H.T., Schmitt, I., Palice, Z., Wiklund, E., Ekman, S., Wedin, M., 2004. Supraordinal phylogenetic relationships of Lecanoromycetes based on a Bayesian analysis of combined nuclear and mitochondrial sequences. Mol. Phylogenet. Evol. 31, 822–832.

Lumbsch, H.T., Wirtz, N., Lindemuth, R., Schmitt, I., 2002. Higher level phylogenetic relationships of euascomycetes (Pezizomycotina) inferred from a combined analysis of nuclear and mitochondrial sequence data. Mycol. Progr. 1 (1), 57–70.

Lutzoni, F., Kauff, F., Cox, C.J., McLaughlin, D., Celio, G., Dentinger, B., Padamsee, M., Hibbett, D., James, T.Y., Baloch, E., Grube, M., Reeb, V., Hofstetter, V., Schoch, C., Arnold, A.E., Miadlikowska, J., Spatafora, J., Johnson, D., Hambleton, S., Crockett, M., Shoemaker, R., Sung, G.-H., Lücking, R., Lumbsch, T., O'Donnell, K., Binder, M., Diederich, P., Ertz, D., Gueidan, C., Hall, B., Hansen, K., Harris, R.C., Hosaka, K., Lim, Y.-W., Liu, Y., Matheny, B., Nishida, H., Pfister, D., Rogers, J., Rossman, A., Schmitt, I., Sipman, H., Stone, J., Sugiyama, J., Yahr, R., Vilgalys, R., 2004. Where are we in assembling the fungal tree of life, classifying the fungi, and understanding the evolution of their subcellular traits. Am. J. Bot.

Lutzoni, F., Pagel, M., Reeb, V., 2001. Major fungal lineages are derived from lichen symbiotic ancestors. Nature 411, 937–940.

Lutzoni, F., Wagner, P., Reeb, V., Zoller, S., 2000. Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. Syst. Biol. 49 (4), 628–651, Program Available from http://www.lutzonilab.net/pages/download.shtml.

Maddison, W.P., Maddison, D.R., 2001. MacClade: Analysis of Phylogeny and Character Evolution, Version 4.01. Sinauer, Sunderland, MA.

Maddison, W.P., Maddison, D.R., 2003. Mesquite: a modular system for evolutionary analysis. Version 1.0 Program. Available from http://mesquiteproject.org.

Magnusson, A.H., 1936. Acarosporaceae und Thelocarpaceae. In: Zahlbruckner, A. (Ed.), Dr. L. Rabenhorst's Kryptogamen—Flora von Deutschland, Österreich und der Schweiz 9 (5, 1). Akademische Verlagsgesellschaft M.B.H., Leipzig, pp. 1–318.

Mason-Gamer, R.J., Kellogg, E.A., 1996. Testing for phylogenetic conflict among molecular data sets in the tribe Triticeae (Gramineae). Syst. Biol. 45 (4), 524–545.

Matheny, P.B., Liu, Y.J., Ammirati, J.F., Hall, B.D., 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (Inocybe, Agaricales). Am. J. Bot. 89 (4), 688–698.

Mau, B., 1996. Bayesian Phylogenetic Inference via Markov Chain Monte Carlo Methods. Ph.D. dissertation, University of Wisconsin, Madison, USA.

Miadlikowska, J., Lutzoni, F., 2004. Phylogenetic classification of peltigeralean fungi (Peltigerales, Ascomycota) based on ribosomal RNA small and large subunits. Am. J. Bot. 91, 449–464.

Miadlikowska, J., Lutzoni, F., Goward, T., Zoller, S., Posada, D., 2003. New approach to an old problem: Incorporating signal from gap-rich regions of ITS and nrDNA large subunit into phylogenetic analyses to resolve the *Peltigera canina* complex. Mycologia 95, 1181–1203.

Miadlikowska, J., McCune, B., Lutzoni, F., 2002. *Pseudocyphellaria perpetua*, a new lichen from Western North America. The Bryologist 105 (1), 1–10.

Myllys, L., Stenroos, S., Thell, A., 2002. New genes for phylogenetic studies of lichenized fungi: glyceraldehyde-3-phosphate dehydrogenase and beta-tubulin genes. Lichenologist 34 (3), 237–246.

Navarro-Rosinés, P., Roux, C., Bellemère, A., 1999. *Thelocarpella gordensis* gen. et sp. nov. (Ascomycetes lichenisati, Acarosporaceae). Can. J. Bot. 77, 835–842.

O'Donnell, K., Lutzoni, F.M., Ward, T.J., Benny, G.L., 2001. Evolutionary relationships among mucoralean fungi (Zygomycota): evidence for family polyphyly on a large scale. Mycologia 93 (2), 286–296.

Ogawa, H., Yoshimura, A., Sugiyama, J., 1997. Polyphyletic origins of species of the anamorphic genus *Geosmithia* and the relationships of the cleistothecial genera: Evidence from 18S, 5S and 28S rDNA sequence analyses. Mycologia 89 (5), 756–771.

Okada, G., Seifert, K.A., Takematsuzz, A., Yamaoka, Y., Miyazaki, S., Tubaki, K., 1998. A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. Can. J. Bot. 76, 1495–1506.

Omland, K.E., 1999. The assumptions and challenges of ancestral state reconstructions. Syst. Biol. 48 (3), 604–611.

Ozenda, P., Clauzade, G., 1970. Les Lichens. Etude Biologique et Flore Illustrée. Masson & Cie, Belgique.

Pagel, M., 1999. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst. Biol. 48, 612–622.

Peršoh, D., Rambold, G., 2002. *Phacopsis*—a lichenicolous genus of the family Parmeliaceae. Mycol. Progr. 1 (1), 43–55.

Platt, J.L., Spatafora, J.W., 2000. Evolutionary relationships of nonsexual lichenized fungi: molecular phylogenetic hypotheses for the genera *Siphula* and *Thamnolia* from SSU and LSU rDNA. Mycologia 92 (3), 475–487.

Poelt, J., 1973. Classification. In: Ahmadjian, V., Hale, M.E. (Eds.), The Lichens. Academic Press, New York, London, pp. 599–632.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Rambold, G., Triebel, D., 1992. The Inter-Lecanoralean Associations. Bibl. Lichenol. 48, Cramer, J., Berlin, Stuttgart.

Rannala, B., Yang, Z., 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol. 43, 304–311.

Roux, C., Triebel, D., 1994. Révision des espèces de *Stigmidium* et de *Sphaerellothecium* (champignons lichénicoles non lichénisés, Ascomycètes) correspondant à *Pharcidia epicymatia* sensu Keissler ou à *Stigmidium schaereri* auct. Bull. Soc. Linn. Provence 45, 451–542.

Roux, C., Triebel, D., Bricaud, O., Le Cœur, D., 1995. Le *Stigmidium lecidellae* sp. nov. et remarques sur le genre *Stigmidium* (champignons lichénicoles non lichénisés, Ascomycètes). Can. J. Bot. 73, 662–672.

Schultz, M., Arendholz, W.-R., Büdel, B., 2001. Origin and evolution of the lichenized ascomycete order Lichinales: monophyly and systematic relationships inferred from ascus, fruiting body and SSU rDNA evolution. Plant Biol. 3, 116–123.

Spatafora, J.W., 1995. Ascomal evolution of filamentous ascomycetes: evidence from molecular data. Can. J. Bot. 73 (Suppl. 1), S811–S815.

Spatafora, J.W., Mitchell, T.G., Vilgalys, R., 1995. Analysis of genes coding for small-subunit rRNA sequences in studying phylogenetics of dematiaceous fungal pathogens. J. Clin. Microbiol. 33 (5), 1322–1326.

Stenroos, S.K., DePriest, P.T., 1998. SSU rDNA phylogeny of cladoniiform lichens. Am. J. Bot. 85 (11), 1548–1559.

Stenroos, S.K., Myllys, L., Thell, A., Hyvönen, J., 2002. Phylogenetic hypotheses: Cladoniaceae, Stereocaulaceae, Baeomycetaceae, and Icmadophilaceae revisited. Mycol. Progr. 1, 267–282.

Suh, S.-O., Blackwell, M., 1999. Molecular phylogeny of the cleistothecial fungi placed in Cephalothecaceae and Pseudodeurotiaceae. Mycologia 91 (5), 836–848.

Suzuki, Y., Glazko, G.V., Nei, M., 2002. Overcredibility of molecular phylogenies obtained by Bayesian phylogenetics. Proc. Natl. Acad. Sci. USA 99 (25), 16138–16143.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, MA.

Tanabe, Y., Saikawa, M., Watanabe, M.M., Sugiyama, J., 2004. Molecular phylogeny of Zygomycota based on EF-1α and RPB1 sequences: limitations and utility of alternative markers to rDNA. Mol. Phylogenet. Evol. 30, 438–449.

Taylor, J.W., Spatafora, J., O'Donnell, K., Lutzoni, F., James, T., Hibbett, D.S., Geiser, D., Bruns, T.D., Blackwell, M., in press. The fungi. In: Cracraft, J., Donoghue, M.J. (Eds.), Assembling the Tree of Life. Oxford University Press.

Tehler, A., 1996. Systematics, phylogeny and classification. In: Nash III, T.H. (Ed.), Lichen Biology. University Press, Cambridge, pp. 217–239.

Tehler, A., Little, D.P., Farris, J.S., 2003. The full-length phylogenetic tree from 1551 ribosomal sequences of chitinous fungi, *Fungi**. Mycol. Res. 107 (8), 901–916.

Terrón, A.A., 2000. The family Fuscideaceae Hafellner in the Iberian Peninsula. The Fourth IAL Symposium, Progress and Problems in Lichenology at the Turn of the Millennium. Universitat de Barcelona, Barcelona. Book of abstracts p. 21.

Thell, A., Stenroos, S., Feuerer, T., Kärnefelt, I., Myllys, L., Hyvönen, J., 2002. Phylogeny of cetrarioid lichens (Parmeliaceae) inferred from ITS and β-tubulin sequences, morphology, anatomy and secondary chemistry. Mycol. Progr. 1 (4), 335–354.

Vězda, A., 1978. Neue oder wenig bekannte Flechten in der Tschechoslowakei. II. Folia Geobot. Phytotax. 13, 397–420.

Waddell, P.J., Kishino, H., Ota, R., 2001. A phylogenetic foundation for comparative mammalian genomics. Genome Informat. 12, 141–154.

Wheeler, W.C., 1990. Combinatorial weights in phylogenetic analysis: a statistical parsimony procedure. Cladistics 6, 269–275.

Wheeler, W.C., Gladstein, D.S., Laet, J.D., Aaronson, L., 2003. POY, ver. 3.0.11a. Program Available from ftp.amnh.org/pub/molecular/poy.

Whittingham, L.A., Slikas, B., Winkler, D.W., Sheldon, F.H., 2002. Phylogeny of the tree swallow genus, *Tachycineta* (Aves: Hirundinidae), by Bayesian analysis of mitochondrial DNA sequences. Mol. Phylogenet. Evol. 22 (3), 430–441.

Wilcox, T.P., Zwickl, D.J., Heath, T.A., Hillis, D.M., 2002. Phylogenetic relationships of the dwarf boas and a comparison of Bayesian and bootstrap measures of phylogenetic support. Mol. Phylogenet. Evol. 25, 361–371.

Yun, S.-H., Berbee, M.L., Yoder, O.C., Turgeon, B.G., 1999. Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. Proc. Natl. Acad. Sci. USA 96, 5592–5597.

Zahlbruckner, A., 1907. Lichenes, Specieller Teil. In: Engler, A., Prantl, K. (Eds.), Die natürlichen Planzenfamilien I (1). Verlag von W. Engelmann, Leipzig, pp. 49–249.