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Implementing a cumulative supermatrix approach for a comprehensive phylogenetic study of the Teloschistales (Pezizomycotina, Ascomycota)

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A B S T R A C T

The resolution of the phylogenetic relationships within the order Teloschistales (Ascomycota, lichen-forming-fungi), with nearly 2000 known species and outstanding phenotypic diversity, has been hindered by the limitation in the resolving power that single-locus or two-locus phylogenetic studies have provided to date. In this context, an extensive taxon sampling within the Teloschistales with more loci (especially nuclear protein-coding genes) was needed to confront the current taxonomic delimitations and to understand evolutionary trends within this order. Comprehensive maximum likelihood and Bayesian analyses were performed based on seven loci using a cumulative supermatrix approach, including protein-coding genes *RPB*1 and *RPB*2 in addition to nuclear and mitochondrial ribosomal RNA-coding genes. We included 167 taxa representing 12 of the 15 genera recognized within the currently accepted Teloschistineae, 22 of the 43 genera within the Physciineae, 49 genera of the closely related orders Lecanorales, Lecideales, and Peltigerales, and the dubiously placed family Brigantiaeaceae and genus *Sipmaniella*. Although the progressive addition of taxa (cumulative supermatrix approach) with increasing amounts of missing data did not dramatically affect the loss of support and resolution, the monophyly of the Teloschistales in the current sense was inconsistent, depending on the loci-taxa combination analyzed. Therefore, we propose a new, but provisional, classification for the re-circumscribed orders Caliciales and Teloschistales (previously referred to as Physciineae and Teloschistineae, respectively). We report here that the family Brigantiaeaceae, previously regarded as *incertae sedis* within the subclass Lecanoromycetidae, and *Sipmaniella*, are members of the Teloschistales in a strict sense. Within this order, one lineage led to the diversification of the mostly epiphytic crustose Brigantiaeaceae and Letrouitiaceae, with a circumpacific center of diversity and found mostly in the tropics. The other main lineage led to another epiphytic crustose family, mostly tropical, and with an Australasian center of diversity – the Megalosporaceae – which is sister to the mainly rock-inhabiting, cosmopolitan, and species rich Teloschistaceae, with a diversity of growth habits ranging from crustose to fruticose. Our results confirm the use of a cumulative supermatrix approach as a viable method to generate comprehensive phylogenies summarizing relationships of taxa with multi-locus to single locus data.

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1. Introduction

Lichens are obligate mutualistic ectosymbioses between fungi (mycobiont) and either or both photobiont green algae and cyanobacteria. Most recent estimates of the number of lichen-forming fungal species have been between 17,500 and 20,000 (i.e., about 20% of all known fungal species; Kirk et al., 2008). This diversity is highly concentrated in the most species rich phylum, the Ascomycota (more specifically within the Leotiomyceta sensu Schoch et al., 2009), with >98% of the lichen-forming fungal species classified in this phylum and accounting for 40% of the phylum species

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richness (Kirk et al., 2008). Within the Leotiomyceta, the great majority of lichen-forming species are found in the third largest class of all Fungi, the Lecanoromycetes. The order Teloschistales as currently delimited, with nearly 2000 known species and accounting for >10% of all known lichen-forming fungi (Kirk et al., 2008), is one of the three main orders recognized within the largest subclass of the Lecanoromycetes – the Lecanoromycetidae (Hibbett et al., 2007; Kirk et al., 2008; Lumbsch and Huhndorf, 2010; Miadlikowska et al., 2006).

The current circumscription of the Teloschistales was established by Miadlikowska et al. (2006) in which two suborders were recognized, the Teloschistineae, which corresponds to the traditionally circumscribed order (composed of the three families Letrouitiaceae, Megalosporaceae, and Teloschistaceae), and the Physciineae, which contains the Caliciaceae (with about 731 species) and the Physciaceae (with 510 species) (Kirk et al., 2008). The recognition of this group of lichens at the ordinal level, and the inclusion of these five families within this order, has been broadly accepted (Hibbett et al., 2007; Kirk et al., 2008; Lumbsch and Huhndorf, 2007).

Growth habits have traditionally played a major role in the classification within these two suborders. Taxa currently included within the Physciineae were initially placed into two separate families, Buelliaceae Zahlbruckner (1907) and Physciaceae Zahlbruckner (1898), based on their respective growth forms, crustose and foliose (Zahlbruckner, 1926b). Subsequently, Poelt (1973) unified both families into the Physciaceae, and Henssen and Jahns (1974) described the suborder Physciineae to encompass the Physciaceae. Ascus and ascospore types have been used traditionally to circumscribe the Physciineae (Bellemère and Letrouit-Galinou, 1981, 1987; Hafellner, 1984; Matzer and Mayrhofer, 1996; Mayrhofer, 1982, 1984). To date, the delimitation of the Caliciaceae and Physciaceae remains controversial. Shared phenotypic similarities by members of these two families and several molecular studies supported a close relationship within the Lecanorales (e.g., Wedin et al., 2000a). The family Physciaceae has been the subject of several articles (e.g., Grube and Arup, 2001; Nordin and Mattsson, 2001; Scheidegger et al., 2001). Based on previous results, Wedin and Grube (2002) proposed the conservation of the family name Physciaceae against Caliciaceae. Later on, Wedin et al. (2002) and Tibell (2003) confirmed that the family Caliciaceae was nested within the Physciaceae *s.l.* (corresponding to the Physciineae). The taxa included in their studies formed two well-supported groups, which corresponded to the informally recognized '*Buellia*-group' and '*Physcia*-group' (Rambold et al., 1994; Wedin et al., 2002). Yet, Helms et al. (2003) formally accepted and emended the family Caliciaceae and the closely related Physciaceae, which corresponded to their clades B and A (i.e., *Buellia*-group and *Physcia*-group), respectively.

Miadlikowska et al. (2006) showed for the first time a sister relationship between the Caliciaceae–Physciaceae (Physciineae) clade and the Letrouitiaceae–Megalosporaceae–Teloschistaceae (Teloschistineae) clade. A large number of new genera have been introduced within the Physciineae (Marbach, 2000). However, it is difficult to assess their relationships and their monophyly without more extensive sampling.

Within the currently accepted Teloschistineae, the monotypic Letrouitiaceae includes about 15 species (Kirk et al., 2008) mostly found in Australia. They are known from tropical to warm-temperate areas, growing on bark, and rarely on decorticated wood and rock (sandstone). The genus *Letrouitia* was established and recognized at the family level based mainly on unique ascus and ascospore structures (Hafellner and Bellemère, 1981c). It seems that within *Letrouitia* a considerable evolution in ascospore septation took place leading to muriform ascospores (Hafellner, 1983; McKillen Burgess, 1963).

The Megalosporaceae was recognized within the Teloschistales (Eriksson, 2005), based on Helms et al. (2003) and Lutzoni et al. (2004). The three genera (*Megalospora*, *Megaloblastenia*, and *Austroblastenia*) classified in this family (with a total of 39 species according to Kirk et al., 2008) are also mostly found in the tropics and subtropics, with a few exceptions in temperate regions, and are corticolous. The family Megalosporaceae was initially proposed by Vězda (1974) to unite *Megalospora* and *Bombyliospora* De Not. with a few other lichen genera. Hafellner and Bellemère (1981a) considered the ascus structure a good character for such a family, but the Megalosporaceae as defined by Vězda was deemed heterogeneous, and Hafellner and Bellemère (1981c) transferred the *Bombyliospora* species with thick spore septa and anthraquinones (i.e., the *B. domingensis* group) to the genus *Letrouitia*. The remaining *Bombyliospora* species were synonymized within *Megalospora* (Hafellner and Bellemère, 1981a).

The family Brigantiaeaceae (Hafellner and Bellemère, 1981b), is currently considered as a family *incertae sedis* within the Lecanoromycetidae, with potentially two genera, *Argopsis*? and *Brigantiaea* (Lumbsch and Huhndorf, 2010) and 25 species (Kirk et al., 2008). Members of this family are found on bark in the tropics and on soil or decaying vegetation in cool climates. The Brigantiaeaceae was initially included within the Lecanorales (Hafellner, 1997), and is still recognized as such by Kirk et al. (2008). *Brigantiaea* species had been placed within the genus *Lopadium* Körb. as circumscribed by Zahlbruckner (1926a,b), which Santesson (1952) found to be heterogeneous. The latter author recognized six species groups within *Lopadium*. One of them, the 'Brigantiaea group,' included species of both the *Lopadium leucoxanthum* group (now *Brigantiaea s.str.*) and the *Bombyliospora domingensis* group (now *Letrouitia*). Due to the presence of muriform ascospores, the latter two groups were considered to be closely related. However, further characters did not support this unification, and *Brigantiaea* was restricted to the *Lopadium leucoxanthum* group (Hafellner, 1997). *Brigantiaea* as circumscribed by Hafellner (1997) seemed to be a well-delimited genus and sufficiently distinct to be recognized at the family level on the basis of ascus structure. Based on those similarities that traditionally linked the above-mentioned genera, we included this family in our study of the Teloschistales with the appropriate set of outgroup taxa to determine its phylogenetic placement within the Lecanoromycetidae.

The family Teloschistaceae, currently with 12 genera and approximately 650 species according to Kirk et al. (2008) (*Caloplaca* [c. 510 spp.], *Cephalophysis* [1 sp.], *Fulgensia* [8 spp.], *Huea* [1 sp.], *Ioplaca* [2 spp.], *Josefpoeltia* [3 spp.], *Seirophora* [11 spp.], *Teloschistes* [33 spp.], *Xanthodactylon* [1 sp.], *Xanthomendoza* [17 sp.], *Xanthopeltis* [1 sp.], and *Xanthoria* [56 spp.]), was first described by Zahlbruckner (1898) to include only foliose and fruticose taxa with polarilocular or 4-locule ascospores. Crustose taxa were placed into a separate family (Caloplacaceae Zahlbruckner, 1926b). Subsequently, Kärnefelt (1989) carried out the most extensive revision of this family and the order Teloschistales *s.str.*, which, with a few exceptions, remains as the main classification in use to date.

Most members of the family Teloschistaceae are easily recognized by the frequent presence of anthraquinones (a secondary metabolite) in apothecial disks, and often in the uppermost layer of the thallus, giving them a yellow to orange color (Santesson, 1970). The Teloschistaceae is a cosmopolitan family, found in most xeric and mesic habitats. The traditional taxonomy within this family was based on vegetative features of the thallus, such as growth form and presence/absence of a lower cortex, and occasionally on secondary substance composition (Søchting, 1997; Søchting and Lutzoni, 2003). Initially, polarilocular ascospores were thought to be a diagnostic trait for this family, but with the inclusion of other genera such as *Cephalophysis*, *Fulgensia*, and *Xanthopeltis*, which have simple or septate spores, the main features defining

the family had to be reconsidered (see Gaya et al., 2008 for more details).

Broad phylogenetic studies within the Lecanoromycetes included only a few specimens from the Teloschistales (e.g., Helms et al., 2003; Lumbsch et al., 2004; Miadlikowska et al., 2006; Miadlikowska and Lutzoni, 2004; Persoh et al., 2004). Studies focusing on the Teloschistaceae, globally or in part, were based on one or two nrDNA loci at the most (e.g., Arup, 2006, 2009; Arup and Grube, 1999; Fedorenko et al., 2009; Gaya et al., 2003, 2008, 2011; Kasalicky et al., 2000; Muggia et al., 2008; Søchting and Lutzoni, 2003; Vondrák et al., 2008, 2009). No phylogenetic study has as yet been designed to sample a representation of taxa across the Teloschistales to confront the current taxonomic delimitations at the family and genus levels. Gaya et al. (2008) published the most exhaustive phylogenetic survey of the Teloschistaceae by restricting the sequencing to ITS and demonstrated that we had reached the limit of the resolving power this single locus can provide. The same is true for phylogenetic studies based on two loci when selected from the nuclear ribosomal tandem repeat. In this context, more loci (especially nuclear protein-coding genes) were needed to enable an extensive taxon sampling within the Teloschistales that could lead to major advancements in their classification and our understanding of evolutionary trends within this order. Consequently, this study sought to provide a multigene phylogeny for the Teloschistales in order to counterpoise the present morphology-based classification with molecular data, and to provide a phylogenetic framework to reassess the currently accepted and also the putative suborders and families (including the Brigantiaeaceae) within the Teloschistales.

2. Materials and methods

2.1. Taxon and character sampling

In this study, we used a cumulative supermatrix approach as in Miadlikowska et al. (2006) to allow a broad and inclusive taxon sampling. Seven loci (detailed below) were used: nuclear 5.8S, small subunit (nucSSU), large subunit (nucLSU), and mitochondrial small subunit (mitSSU) ribosomal RNA-coding genes, as well as two nuclear protein-coding genes, *RPB*1 (one locus) and *RPB*2 (two amplicons, considered here as two separate loci in terms of analyses). A dataset of 45 taxa with all seven loci was initially assembled; this 7-locus dataset was enlarged with taxa for which at least six of the seven-targeted loci were available, resulting in a 7 + 6-locus dataset of 85 taxa. Subsequently, taxa with at least five loci were added to form a 7 + 6 + 5-locus dataset of 107 taxa. This process was repeated for taxa with at least 4 loci (total of 123 taxa, 7 + 6 + 5 + 4-locus dataset), 3 loci (total of 147 taxa, 7 + 6 + 5 + 4 + 3-locus dataset), 2 loci (total of 160 taxa, 7 + 6 + 5 + 4 + 3 + 2-locus dataset), and 1 locus (total of 167 taxa, 7 + 6 + 5 + 4 + 3 + 2 + 1-locus dataset), for a total of seven different taxon sets (with an increasing amount of missing data) that were analyzed separately (Table 1).

The largest dataset of 167 taxa (7 + 6 + 5 + 4 + 3 + 2 + 1-locus dataset) includes 78 taxa representing the three recognized families within the suborder Teloschistineae (the Letrouitiaceae [6 species, 1 genus], the Megalosporaceae [8 species, 3 genera], and the Teloschistaceae [58 species, 9 genera]), and one family of unknown placement in the Lecanoromycetidae (the Brigantiaeaceae [6 species, 1 genus]); the suborder Physciineae with representative taxa from the families Caliciaceae and Physciaceae (39 taxa, 22 genera); and three outgroup orders: the Lecanorales (34 species, 34 genera), the Peltigerales (13 species, 13 genera), and the Lecideales (3 species, 2 genera), selected based on Miadlikowska et al. (2006). This taxon sampling represents 63% of genera and 5.6% of species currently accepted within the Teloschistales.

Genomic DNA was obtained from fresh samples and herbarium specimens (voucher information is detailed in Supplementary material 1). From a total of 832 sequences included in this study, 332 (40%) are published here for the first time, and the rest were obtained from GenBank and the AFTOL database (see Supplementary material 1 for GenBank accession or ID numbers).

2.2. Molecular data

The seven regions targeted for this study were: ≈0.6 kb of ITS region for the 5.8S using primers ITS1F–ITS4 (Gardes and Bruns, 1993; White et al., 1990), ≈1.6 kb at the 5′ end of the nucSSU using primers nssu131–NS24 as well as nssu897R, nssu1088R, SR7R, nssu634, nssu1088, and SR7 (Gargas and Taylor, 1992; Kauff and Lutzoni, 2002; R. Vilgalys web site), ≈1.4 kb at the 5′ end of nucLSU using primers LR0R (or LIC24R)–LR7 as well as LR3R and LR3 (Miadlikowska and Lutzoni, 2000; Rehner and Samuels, 1994; Vilgalys and Hester, 1990), ≈0.8 kb of mitSSU using primers mitSSU1–mitSSU3R and mitSSU2R (Zoller et al., 1999), ≈1–1.2 kb of *RBP*1 using primers *RPB*1-A_f (or *RPB*1-AFasc)–*RPB*1–6R1asc (or *RPB*1–6R2asc) (region A–D; Hofstetter et al., 2007; Stiller and Hall, 1997), ≈0.8–1.0 kb of *RPB*2 using primers *RPB*2–980F (or f*RPB*2–5F)–f*RPB*2–7cR as well as *RPB*2–1554F and *RPB*2–1554R (region 5–7; Liu et al., 1999; Reeb et al., 2004), and ≈0.9–1.0 kb of *RPB*2 using primers f*RPB*2–7cF–f*RPB*2–11aR as well as *RPB*2–3053R and *RPB*2–3053bR (region 7–11; Liu et al., 1999; Reeb et al., 2004). These primers can be found at http://www.lutzonilab.net/primers/,

Table 1
Synopsis of number of OTUs, alignment lengths, number of analyzed characters, and number of constant and variable characters included for each locus separately and when combined in the seven supermatrices.

Loci	OTU	Alignment length	Included char.	Constant char.	Variable char.
5.8S	143	154	151	89	62
mitSSU	143	1365	524	282	242
nucLSU	133	3976	1149	719	430
nucSSU	140	8471	1555	1111	444
*RPB*1 [A–D]	92	1284	1023	425	598
*RPB*2 [5–7]	83	1164	993	375	618
*RPB*2 [7–11]	100	957	912	438	474
Combined datasets					
7-locus	45	17,371	6307	3869	2438
7 + 6-locus	85	17,371	6307	3606	2701
7 + 6 + 5-locus	107	17,371	6307	3494	2813
7 + 6 + 5 + 4-locus	123	17,371	6307	3463	2844
7 + 6 + 5 + 4 + 3-locus	147	17,371	6307	3432	2875
7 + 6 + 5 + 4 + 3 + 2-locus	160	17,371	6307	3427	2880
7 + 6 + 5 + 4 + 3 + 2 + 1-locus	167	17,371	6307	3427	2880

www.aftol.org/data.php, and http://www.biology.duke.edu/fungi/mycolab/primers.htm.

Genomic DNA was extracted from dried material or cultures using a protocol modified from Zolan and Pukkila (1986) with 2% sodium dodecyl sulfate (SDS) as the extraction lysis buffer. PVPP (polyvinyl polypyrolidone) was added to remove pigments and phenolic compounds that could potentially interfere with DNA isolation and PCR reaction. After precipitating the genomic DNA using isopropanol, pellets were washed once in 70% ethanol, dried with a speedvac, and resuspended in 30–50 ml sterile water and stored at −20 °C. Protocols for the amplification of the targeted loci can be found in Hofstetter et al. (2007), James et al. (2006), and Lutzoni et al. (2004). After examination with gel electrophoresis, PCR products were purified using the Microcon PCR cleaning kit (Millipore, Billerica, MA) or ExoSAP-IT® (USB Corporation, Cleveland, OH). Alternatively, cloning was conducted on weak PCR products, PCR products presenting multiple bands, and most of the PCR products of *RPB*1 and *RPB*2, using the TOPO TA Cloning® Kit (Invitrogen™, Life Technologies, Carlsbad, CA). Sequencing was carried out in 10 μl reactions using: 1 μl primer, 1 μl purified PCR product, 0.75 μl Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin–Elmer, Applied Biosystems, Foster City, CA), 3.25 μl Big Dye buffer, and 4 μl double-distilled water. Automated reaction clean up and visualization was performed at the Duke Genome Sequencing & Analysis Core Facility of the Institute for Genome Sciences and Policies. Clean up reactions were performed over Sephadex G-50 DNA grade columns, eluting in water. Samples were then injected directly on an ABI 3730xl DNA analyzer (PE Applied Biosystems, Foster City, CA) utilizing a 22 s injection time, and a 50 cm capillary array.

2.3. Sequence alignment

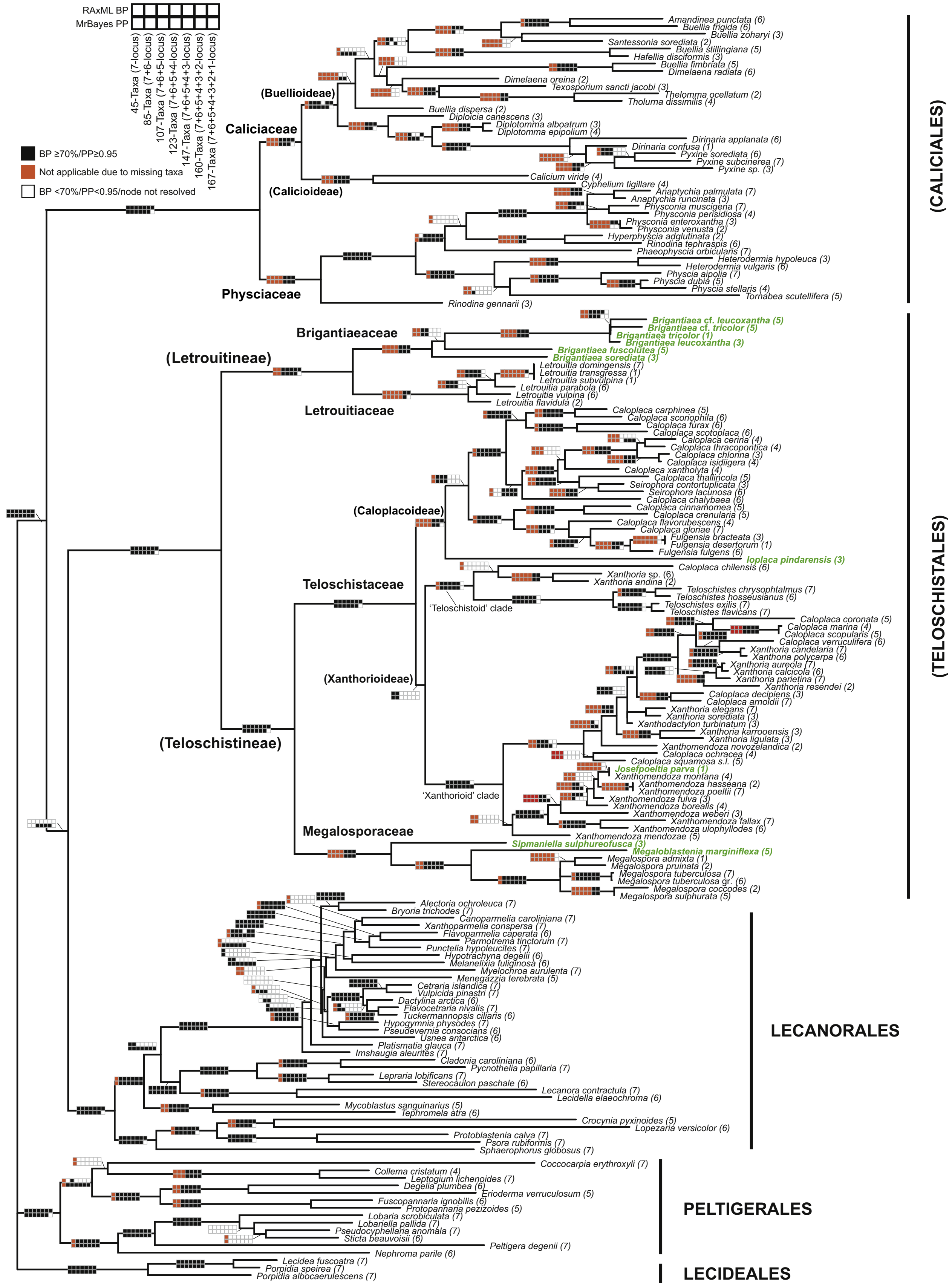
Sequence fragments were subjected to BLAST searches for a first verification of their identities. They were assembled and edited using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI), and aligned manually with MacClade 4.08 (Maddison and Maddison, 2005). Following Kjer (1995), the nucSSU, nucLSU and mitSSU sequences were aligned with the help of the secondary structure of these RNA molecules obtained from *Saccharomyces cerevisiae* (for nucSSU and nucLSU), and from *Emericella nidulans* (for mitSSU), as reported by Cannone et al. (2002). Ambiguously aligned regions (sensu Lutzoni et al., 2000) and introns were delimited manually and excluded from subsequent analyses. All new sequences were deposited in GenBank (Supplementary material 1) and the concatenated alignment of seven loci was deposited in TreeBASE (accession number 12226).

2.4. Phylogenetic analyses

Topological incongruence among each locus separately and on all possible combinations (42) of the seven loci was examined with 1000 replicates of ML bootstrapping (ML-BS) and the GTRGAMMA model using RAxML-VI-HPC (Stamatakis, 2006). A conflict was assumed to be significant if two different relationships (one being monophyletic and the other being non-monophyletic) for the same set of taxa were both supported with bootstrap values ⩾70% (Mason-Gamer and Kellogg, 1996). For the *RPB*2 locus, this criterion was applied on each amplicon separately. If no conflict was detected, loci were concatenated.

Phylogenetic relationships and confidence were inferred using maximum likelihood (ML) for each supermatrix. Additional support values were estimated using a Bayesian approach. For the ML analyses, all seven supermatrices were divided into 13 partitions (5.8S, nucSSU, nucLSU, mitSSU, *RPB*1/1st, 2nd, 3rd, *RPB*2–1st amplicon/1st, 2nd, 3rd, and *RPB*2–2nd amplicon/1st, 2nd, 3rd). The program RAxML-VI-HPC (Stamatakis, 2006) was used to estimate the most likely tree with 1000 replicates and a GTRGAMMA model of molecular evolution. Bootstrap proportions (ML-BS) were obtained from 1000 replicates of ML bootstrapping conducted with the same settings and program. For the Bayesian analyses, supermatrices were divided into seven partitions with 1st, 2nd, and 3rd codon positions linked across *RPB*1 and *RPB*2 in order to avoid overparameterization (Chang, 1996; McGuire et al., 2007; Rannala, 2002; Steel, 2005) suggested by poor convergence of MCMC chains when using 13 partitions in our analyses performed with MrBayes 3.1.2 (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2002; Ronquist and Huelsenbeck, 2003). Some of the features of MCMC analyses that have been proposed in the literature (Castoe et al., 2004) to be monitored to identify overparameterization are poor convergence of MCMC chains (Carlin and Louis, 1996), delayed convergence of an MCMC chain (Rannala, 2002), failure of multiple independent chains of the same model to converge on similar estimates of parameters and posterior probabilities (Huelsenbeck et al., 2002; see also Strugnell et al., 2005, and Nylander et al., 2004), as well as inaccurate branch length estimates as a result of poor mixing or posterior distributions with excessive weight at long tree lengths, especially accute in parameter rich models (Brown et al., 2010; Ekman and Blaalid, 2011; Marshall, 2010). The AIC in MrModeltest 2.3 (Nylander, 2004) was used to choose the model of molecular evolution for each partition. A GTR (Rodriguez et al., 1990) model with an estimated proportion of invariable sites and a gamma distribution approximated with four categories was used for all partitions, except for 5.8S where a SYM (Zharkikh, 1994) model, also with an estimated proportion of invariable sites and a gamma distribution approximated with four categories, was selected. Prior distributions included a (1, 1, 1, 1, 1, 1) Dirichlet for the substitution rate, a (1, 1, 1, 1) Dirichlet for the state frequencies, a uniform (0, 200) distribution for the gamma shape parameter, a uniform (0, 1) distribution for the proportion of invariable sites, a uniform for topologies, and an exponential (10) distribution for branch lengths in all partitions. Two analyses of four chains were run for 20 M (7-locus and 7 + 6-locus datasets) and 50 M (the remaining datasets) generations using MrBayes 3.1.2, with trees sampled every 500 generations. The log-likelihood scores were graphically explored by plotting them against generation time with Tracer v1.4.1 (Rambaut and Drummond, 2007; http://beast.bio.ed.ac.uk/Tracer) and set stationarity when log-likelihood values reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001) and when average standard deviation of split frequencies across runs dropped below 0.01. This was also verified with the AWTY program (Wilgenbusch et al., 2004; http://ceb.csit.fsu.edu/awty; Nylander et al., 2008). A burn-in sample of 12,000 trees (7-locus and 7 + 6-locus datasets), 40,000 trees (7 + 6 + 5 + 4 + 3 + 2 + 1-locus dataset), and 30,000 trees (the remaining datasets) was discarded for each run. The remaining 56,000 trees (7-locus and 7 + 6-locus datasets), 120,000 trees (7 + 6 + 5 + 4 + 3 + 2 + 1-locus dataset), and 140,000 trees (the remaining datasets) were used to estimate branch lengths with the sumt command in MrBayes, and Posterior Probabilities (PP) with the majority rule consensus tree command in PAUP* 4.0b10 (Swofford, 2002). Internodes with bootstrap proportions ⩾70% and Bayesian posterior probabilities ⩾0.95 were considered strongly supported (Fig. 1). Internodes with a bootstrap value ⩾70% and a posterior probability <0.95 were also interpreted as well supported (Alfaro et al., 2003; Lutzoni et al., 2004). Constant sites were included for all ML and Bayesian analyses.

Additionally, the resulting trees from the 1000 replicates of ML bootstrapping conducted on each locus separately were used to explore the support for specific internodes, i.e., the sister clade to the Teloschistineae (sensu Miadlikowska et al., 2006), referred to in this study as Teloschistales. In PAUP, we loaded a constraint-tree with all internodes collapsed except for the one of interest, and then we used it as a filter when loading the 1000 bootstrap trees, for all seven sets



RAxML BP
MrBayes PP
45-Taxa (7-locus)
85-Taxa (7+6-locus)
107-Taxa (7+6+5-locus)
123-Taxa (7+6+5+4-locus)
147-Taxa (7+6+5+4+3-locus)
160-Taxa (7+6+5+4+3+2-locus)
167-Taxa (7+6+5+4+3+2+1-locus)
BP ≥70%/PP≥0.95
Not applicable due to missing taxa
BP <70%/PP<0.95/node not resolved
Caliciaceae
(Buellioideae)
(Calicioideae)
Physciaceae
Brigantiaeaceae
(Letrouitineae)
Letrouitiaceae
(Caloplacoideae)
Teloschistaceae
'Teloschistoid' clade
(Xanthorioideae)
(Teloschistineae)
'Xanthorioid' clade
Megalosporaceae
(CALICIALES)
(TELOSCHISTALES)
LECANORALES
PELTIGERALES
LECIDEALES
Amandinea punctata (6)
Buellia frigida (6)
Buellia zoharyi (3)
Santessonia sorediata (2)
Buellia stillingiana (5)
Hafellia disciformis (3)
Buellia fimbriata (5)
Dimelaena radiata (6)
Dimelaena oreina (2)
Texosporium sancti jacobi (3)
Thelomma ocellatum (2)
Tholurna dissimilis (4)
Buellia dispersa (2)
Diploicia canescens (3)
Diplotomma alboatrum (3)
Diplotomma epipolium (4)
Dirinaria applanata (6)
Dirinaria confusa (1)
Pyxine sorediata (6)
Pyxine subcinerea (7)
Pyxine sp. (3)
Calicium viride (4)
Cyphelium tigillare (4)
Anaptychia palmulata (7)
Anaptychia runcinata (3)
Physconia muscigena (7)
Physconia perisidiosa (4)
Physconia enteroxantha (3)
Physconia venusta (2)
Hyperphyscia adglutinata (2)
Rinodina tephraspis (6)
Phaeophyscia orbicularis (7)
Heterodermia hypoleuca (3)
Heterodermia vulgaris (6)
Physcia aipolia (7)
Physcia dubia (5)
Physcia stellaris (4)
Tornabea scutellifera (5)
Rinodina gennarii (3)
Brigantiaea cf. leucoxantha (5)
Brigantiaea cf. tricolor (5)
Brigantiaea tricolor (1)
Brigantiaea leucoxantha (3)
Brigantiaea fuscolutea (5)
Brigantiaea sorediata (3)
Letrouitia domingensis (7)
Letrouitia transgressa (1)
Letrouitia subvulpina (1)
Letrouitia parabola (6)
Letrouitia vulpina (6)
Letrouitia flavidula (2)
Caloplaca carphinea (5)
Caloplaca scoriophila (6)
Caloplaca furax (6)
Caloplaca scotoplaca (6)
Caloplaca cerina (4)
Caloplaca thracopontica (4)
Caloplaca chlorina (3)
Caloplaca isidiigera (4)
Caloplaca xantholyta (4)
Caloplaca thallincola (5)
Seirophora contortuplicata (3)
Seirophora lacunosa (6)
Caloplaca chalybaea (6)
Caloplaca cinnamomea (5)
Caloplaca crenularia (5)
Caloplaca flavorubescens (4)
Caloplaca gloriae (7)
Fulgensia bracteata (3)
Fulgensia desertorum (1)
Fulgensia fulgens (6)
Ioplaca pindarensis (3)
Caloplaca chilensis (6)
Xanthoria sp. (6)
Xanthoria andina (2)
Teloschistes chrysophtalmus (7)
Teloschistes hosseusianus (6)
Teloschistes exilis (7)
Teloschistes flavicans (7)
Caloplaca coronata (5)
Caloplaca marina (4)
Caloplaca scopularis (5)
Caloplaca verruculifera (6)
Xanthoria candelaria (7)
Xanthoria polycarpa (6)
Xanthoria aureola (7)
Xanthoria calcicola (6)
Xanthoria parietina (7)
Xanthoria resendei (2)
Caloplaca decipiens (3)
Caloplaca arnoldii (7)
Xanthoria elegans (7)
Xanthoria sorediata (3)
Xanthodactylon turbinatum (3)
Xanthoria karrooensis (3)
Xanthoria ligulata (3)
Xanthomendoza novozelandica (2)
Caloplaca ochracea (4)
Caloplaca squamosa s.l. (5)
Josefpoeltia parva (1)
Xanthomendoza montana (4)
Xanthomendoza hasseana (2)
Xanthomendoza poeltii (7)
Xanthomendoza fulva (3)
Xanthomendoza borealis (4)
Xanthomendoza weberi (3)
Xanthomendoza fallax (7)
Xanthomendoza ulophyllodes (6)
Xanthomendoza mendozae (5)
Sipmaniella sulphureofusca (3)
Megaloblastenia marginiflexa (5)
Megalospora admixta (1)
Megalospora pruinata (2)
Megalospora tuberculosa (7)
Megalospora tuberculosa gr. (6)
Megalospora coccodes (2)
Megalospora sulphurata (5)
Alectoria ochroleuca (7)
Bryoria trichodes (7)
Canoparmelia caroliniana (7)
Xanthoparmelia conspersa (7)
Flavoparmelia caperata (6)
Parmotrema tinctorum (7)
Punctelia hypoleucites (7)
Hypotrachyna degelii (6)
Melanelixia fuliginosa (6)
Myelochroa aurulenta (7)
Menegazzia terebrata (5)
Cetraria islandica (7)
Vulpicida pinastri (7)
Dactylina arctica (6)
Flavocetraria nivalis (7)
Tuckermannopsis ciliaris (6)
Hypogymnia physodes (7)
Pseudevernia consocians (6)
Usnea antarctica (6)
Platismatia glauca (7)
Imshaugia aleurites (7)
Cladonia caroliniana (6)
Pycnothelia papillaria (7)
Lepraria lobificans (7)
Stereocaulon paschale (6)
Lecanora contractula (7)
Lecidella elaeochroma (6)
Mycoblastus sanguinarius (5)
Tephromela atra (6)
Crocynia pyxinoides (5)
Lopezaria versicolor (6)
Protoblastenia calva (7)
Psora rubiformis (7)
Sphaerophorus globosus (7)
Coccocarpia erythroxyli (7)
Collema cristatum (4)
Leptogium lichenoides (7)
Degelia plumbea (6)
Erioderma verruculosum (5)
Fuscopannaria ignobilis (6)
Protopannaria pezizoides (5)
Lobaria scrobiculata (7)
Lobariella pallida (7)
Pseudocyphellaria anomala (7)
Sticta beauvoisii (6)
Peltigera degenii (7)
Nephroma parile (6)
Lecidea fuscoatra (7)
Porpidia speirea (7)
Porpidia albocaerulescens (7)
0.05 substitutions/site

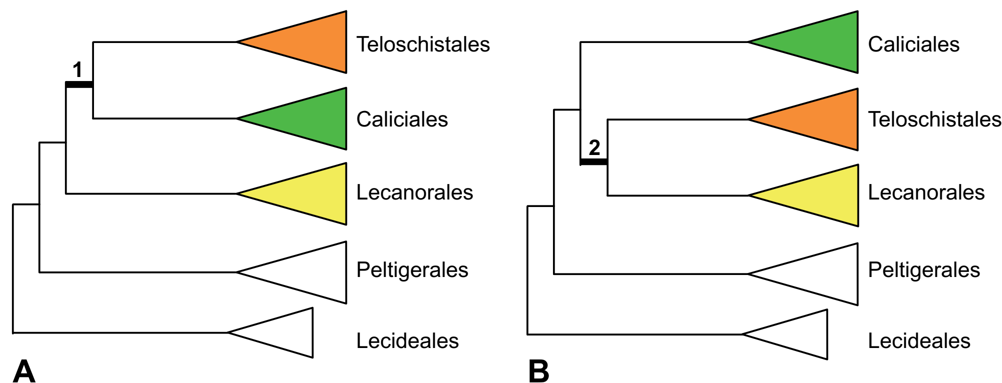


Fig. 2. (A and B) Schematic trees showing the two alternative relationships revealed by the different analyses performed on various datasets. (A) The Teloschistales appears as sister to the Caliciales. (B) The Teloschistales is sister to the Lecanorales. Numbers 1 and 2 indicate the alternative topological resolution.

of 1000 bootstrap replicates, with the aim of retaining only those containing that internode. We repeated this operation twice, with two different constraint-trees: one with the Caliciales (Physciineae sensu Miadlikowska et al., 2006) sister to the Teloschistales and a second with the Lecanorales sister to the Teloschistales (Fig. 2).

3. Results

3.1. Alignments

A synopsis of datasets used in our analyses, including alignment lengths, number of included sites, and missing sequences per locus and dataset is shown in Tables 1 and 2. Ribosomal RNA-coding genes contained many indels. The nucSSU and nucLSU were particularly rich in group I and spliceosomal introns, especially in the Caliciales and Teloschistales. From the RNA polymerase II genes, only *RPB*1 hosted one spliceosomal intron. As expected, *RPB*1 and *RPB*2 loci showed the highest proportion of unambiguously aligned sites included in the phylogenetic analyses (∼78–95%) compared to the ribosomal genes (∼18–38%), with the exception of the short 5.8S (98%) that contributed minimally to the number of variable sites with 60% of its 151 included sites being constant. The proportion of missing data went from 6.7% in the 7 + 6-locus dataset to 28.7% in the largest dataset (167 taxa), due mostly to missing *RPB*1 and *RPB*2 sequences (Table 2). Therefore, the loci with the largest amount of missing sequences are the same loci contributing the largest number of unambiguously aligned variable sites: 84 missing sequences for *RPB*2 [5–7] out of 167, with 618 variable sites; 75 missing sequences for *RPB*1 out of 167, with 598 variable sites; 67 missing sequences for *RPB*2 [7–11] out of 167, with 474 variable sites (Tables 1 and 2). The nucSSU was the locus with the least amount of missing sequences (27/167) that contributed the highest number of variable sites (444). The nucLSU was the second best locus in this regard.

3.2. Cumulative supermatrix approach

Except for a specific internode (Fig. 2A and B) discussed below, the supermatrix approach adopted here did not generate conflictual phylogenetic relationships (Miadlikowska et al., 2006; Wiens, 2006). Topologies recovered from progressively larger datasets with higher numbers of species and increasing amount of missing data, were consistent with the 7-locus phylogeny (smallest dataset in terms of number of species and no missing sequences). Overall, most relationships were well supported by most datasets and methods (ML and Bayesian approach), and a few branches were confidently recovered only by the most complete datasets (7-locus, 7 + 6-locus). Adding species with only one or two loci (7 + 6 + 5 + 4 + 3 + 2-locus and 7 + 6 + 5 + 4 + 3 + 2 + 1-locus datasets) allowed the phylogenetic placement of several additional species with confidence (e.g., within the *Brigantiaea–Letrouitia* and *Megalosporaceae* clades), but in general caused support values to be lower (Fig. 1). Comparing the optimization methods, maximum likelihood and Bayesian analyses provided similar support and resolution (Fig. 1, the most likely tree from the ML analysis on the largest dataset, 7 + 6 + 5 + 4 + 3 + 2 + 1-locus, is shown). However, Bayesian analyses presented certain difficulties in reaching convergence when adding more taxa with missing data, and in recovering long tree lengths estimates. This problem was especially acute when unlinking all 13 partitions probably due to a phenomenon of overparameterization mixed with a higher sensitivity of the Bayesian approach to so many missing data, as current Bayesian programs tend to mix very slowly when analyzing datasets with large proportions of missing data (Ané et al., 2007). For this reason, we decided to link the three-codon positions across the protein-coding genes and let the program estimate the model parameters (D. Swofford, pers. comm.). In doing so, Bayesian Markov chains reached convergence. An additional option to overcome poor mixing would have been to start MCMC analyses from trees with shorter branches than the default (Brown et al., 2010; Ekman and Blaalid, 2011; Marshall, 2010). After fixing the convergence problem, we observed an increase in tree length when more taxa and more missing data were added in the Bayesian analyses compared to ML estimates. Whereas in the 7-locus analyses both optimization methods recovered a similar tree length (2.5 for ML versus 2.6 for the Bayesian approach), in the largest dataset the Bayesian tree length nearly doubled the ML estimate (10.9 and

Fig. 1. Most likely tree (−ln likelihood = −122411.558258) depicting phylogenetic relationships among 78 members of the Teloschistales, 39 of the Caliciales, and 34 of the Lecanorales based on a combined 5.8S, nucSSU, nucLSU, mitSSU, *RPB*1 and *RPB*2 (two loci) supermatrix (7 + 6 + 5 + 4 + 3 + 2 + 1-locus dataset). Sixteen species from the Peltigerales and Lecideales were used to form the outgroup. Numbers in parentheses after species names indicate the number of loci for which DNA sequences were available for that species. The 14-box grids on internodes show support with different phylogenetic methods and datasets. Top row boxes indicate bootstrap values calculated with RAxML and bottom row boxes indicate posterior probabilities calculated with MrBayes on each of the seven datasets with different number of loci, taxa, and proportion of missing data (Tables 1 and 2). Red boxes indicate cases where internodal support is not applicable due to the absence of that internode resulting from the absence of at least one of the two derived lineages for a specific analysis, compared to the largest 167 taxa dataset. Black boxes indicate RAxML bootstrap values ⩾70% or MrBayes posterior probability values ⩾0.95. White boxes indicate RAxML bootstrap values <70%, or MrBayes posterior probability values <0.95. In green are highlighted the genera included within a molecular phylogenetic study for the first time. Names in quotes refer to rank-less taxonomic entities proposed here for the first time. Names in parentheses are provisionary taxonomic names proposed here to ultimately forge a new, phylogenetically based, classification for the Teloschistales and Caliciales. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Synopsis table of missing sequences per locus and dataset. For each locus, absolute numbers of missing sequences (first column), percentage of missing sequences with respect to the total number of sequences per dataset (second column), and percentage of missing sequences with respect to the total number of sequences per locus in each dataset (third column) are shown. The last two columns of this table show the total number of missing sequences per dataset and its corresponding percentage.

Datasets	5.8S			mitSSU			nucLSU			nucSSU			*RPB*1			*RPB*2 [5–7]			*RPB*2 [7–11]			Missing seq.	Percent total missing seq.
45-Taxon	0	0%	0%	0	0%	0%	0	0%	0%	0	0%	0%	0	0%	0%	0	0%	0%	0	0%	0%	0	0
85-Taxon	4	0.70%	4.70%	3	0.50%	3.50%	0	0%	0%	4	0.70%	4.70%	11	1.80%	12.90%	12	2%	14.10%	6	1%	7.10%	40	6.70%
107-Taxon	11	1.50%	10.30%	6	0.80%	5.60%	2	0.30%	1.90%	8	1%	7.50%	17	2.30%	15.90%	27	3.6%,	25.20%	13	1.70%	12.10%	84	11.20%
123-Taxon	13	1.50%	10.60%	6	0.70%	4.90%	3	0.30%	2.40%	11	1.30%	8.90%	32	3.70%	26%	42	4.90%	34.10%	25	2.90%	20.30%	132	15.30%
147-Taxon	18	1.70%	12.20%	11	1.20%	7.50%	17	1.70%	11.60%	16	1.60%	10.90%	55	5.30%	37.40%	64	6.20%	43.50%	47	4.60%	32%	228	22.20%
160-Taxon	21	1.90%	13.10%	19	1.70%	11.90%	27	2.40%	16.90%	21	1.90%	13.10%	68	6%	42.50%	77	6.90%	48.10%	60	5.50%	37.50%	293	26.20%
167-Taxon	24	2.10%	14.40%	24	2.10%	14.40%	34	2.90%	20.40%	27	2.30%	16.20%	75	6.40%	44.90%	84	7.20%	50.30%	67	5.70%	40.10%	335	28.70%

5.6, respectively). The Bayesian analysis took much longer to converge for the largest dataset (167 taxa) than for the others, and for many internodes the ML analysis was more efficient in resolving internodes and recovering high support than the Bayesian analysis on that dataset. Although the Bayesian analysis resolved most internodes, it did not recover posterior probabilities $\geqslant 0.95$ for many of them (see Fig. 1, last square at the bottom right).

The supermatrix approach led to conflicting results only on a specific relationship (Fig. 2A and B), this is the sister clade to the Teloschistales. The ML bootstrap analyses performed on each locus separately to determine the proportion of bootstrap trees per locus that recovered the two alternative relationships are summarized in Table 4. Bootstrap analyses of the nucSSU, nucLSU, and *RPB*1 generated a higher number of trees with the Caliciales as sister to the Teloschistales, whereas the two amplicons of *RPB*2 and mitSSU generated a higher number of trees with the Lecanorales as the closest relative to Teloschistales (Table 4; see Section 4).

3.3. Phylogenetic relationships

The two suborders Teloschistineae and Physciineae sensu Miadlikowska et al. (2006), here treated as orders Teloschistales and Caliciales, appeared well supported as sister groups when analyzing the dataset with no missing data (7-locus, i.e., 45-taxon, dataset) both with ML BP and PP (Fig. 2A and Table 3). The same relationship was found with the 7 + 6-locus (i.e., 85-taxon) dataset, but only with ML and a low bootstrap value. The phylogenetic searches on the remaining datasets recovered the Lecanorales sister to the Teloschistales (Fig. 2B and Table 3), but never significantly supported with ML BP. However, this alternative relationship received posterior probabilities >0.95 for the 7 + 6 + 5-locus to the 7 + 6 + 5 + 4 + 3 + 2-locus datasets (Table 3).

Within the Teloschistales (Fig. 1), all relationships among major groups were well supported, including lineages that had never been included in earlier phylogenetic studies. Our analyses place the family Brigantiaeaceae within the Teloschistales with strong support for sharing a most recent common ancestor with the Letrouitiaceae. The Brigantiaeaceae–Letrouitiaceae clade is henceforth referred to as suborder Letrouitineae (Fig. 1). The other main lineage within the Teloschistales gave rise to the Megalosporaceae and Teloschistaceae, henceforth referred to as suborder Teloschistineae (Fig. 1). The family Megalosporaceae is represented by three genera, *Megaloblastenia*, *Megalospora*, and *Sipmaniella*. Therefore, the phylogenetic placement of the recently described monotypic genus *Sipmaniella* confirms that *S. sulphureofusca* does not belong to the genus *Lecania* (Kalb et al., 2009; Reese Næsborg et al., 2007), but also reveals that it is not part of the family Lecanoraceae, where *Sipmaniella* is currently classified (Lumbsch and Huhndorf, 2010), and further expands the Megalosporaceae (Fig. 1).

Table 3
Bootstrap proportions (BP) and Bayesian posterior probabilities (PP) recovered depending on the dataset analyzed for the two alternative sister relationships shown in Fig. 2: the Caliciales sister to the Teloschistales (relationship 1) or the Lecanorales sister to the Teloschistales (relationship 2). BP > 70% and PP > 0.95 are in bold. NA = Not applicable.

Datasets	ML analyses (BP)		Bayesian analyses (PP)	
	Caliciales (1) (%)	Lecanorales (2) (%)	Caliciales (1)	Lecanorales (2)
45-Taxon	**73**		**1**	
85-Taxon	51			0.64
107-Taxon		55		**0.98**
123-Taxon		55		**0.99**
147-Taxon		52		**0.99**
160-Taxon		53		**0.99**
167-Taxon		48	NA	NA

All analyses supported with high confidence the monophyly of the Teloschistaceae, which includes in this study *Caloplaca*, *Fulgensia*, *Ioplaca*, *Josefpoeltia*, *Seirophora*, *Teloschistes*, *Xanthodactylon*, *Xanthomendoza*, and *Xanthoria*. In general, most phylogenetic relationships among genera and species groups within the Teloschistaceae were confidently recovered. For instance, the genus *Teloschistes* was here well supported as monophyletic for the first time, and was recovered with confidence as sister to two South American taxa – *Caloplaca chilensis* and *Xanthoria andina*. This sister relationship (referred to here as the 'Teloschistoid' clade; Fig. 1) diverged from a lineage that led to a clade populated mostly by lobed anthraquinone-containing species of *Caloplaca*, *Xanthomendoza*, and *Xanthoria* (referred hereafter as the 'Xanthorioid' clade). The 'Teloschistoid' and 'Xanthorioid' clades are here considered within the subfamily Xanthorioideae. The first divergence within the 'Xanthorioid' clade leads to the well-supported monophyletic genus *Xanthomendoza*, with the exception of *X. novozelandica*, and the inclusion of *Josefpoeltia*. The other lineage resulting from this split encompasses most species of the genus *Xanthoria*, several *Caloplaca* species, one representative of the genus *Xanthodactylon*, and *Xanthomendoza novozelandica*. Both *Caloplaca* and *Xanthoria* were recovered here as polyphyletic.

Within the phenotypically diverse clade populated by *Caloplaca* species that mostly lack anthraquinones in their thallus, referred to here as the subfamily Caloplacoideae (Fig. 1), the genera *Fulgensia* and *Seirophora* were significantly recovered as monophyletic. With a remarkably long branch, the genus *Ioplaca* is derived from the first split within this subfamily.

Regarding the Caliciales, many relationships at the genus level received high bootstrap support and the same two sister groups, referred to here as the families Caliciaceae (previously the buellioid clade or *Buellia* group) and Physciaceae (previously the rinodinoid clade or *Physcia* group), were recovered as in previous studies (Fig. 1) (e.g., Helms et al., 2003; Miadlikowska et al., 2006). Within the Physciaceae, *Anaptychia*, *Heterodemia*, and *Physcia* were recovered as well-supported monophyletic genera. In the Caliciaceae, some of the taxa traditionally considered part of the family Caliciaceae *s.str.* appeared as monophyletic in an early diverging clade composed here of *Calicium* and *Cyphelium*, and referred to therein as the Calicioideae, while *Texosporium*, *Thelomma*, and *Tholurna* formed a monophyletic group within the sister subfamily Buellioideae. The genera *Buellia* and *Dimelaena* appeared polyphyletic, whereas the included representatives of *Diplotomma* and *Pyxine* were recovered as monophyletic genera, and share a most recent common ancestor with *Dirinaria* and *Diploicia*.

4. Discussion

4.1. Inconsistent phylogenetic signal for the monophyly of the Teloschistales s.l.

One of our goals for this study was to assess the sister relationship between the Teloschistales *s.str.* and Caliciales (Fig. 2A), or what was considered the monophyletic order Teloschistales by Miadlikowska et al. (2006), i.e., Teloschistineae sister to Physciineae. We encountered several issues in that matter, as this was the only case where the cumulative supermatrix approach led to conflicting results. Before this study, only Miadlikowska et al. (2006) and Miadlikowska and Lutzoni (2004) recovered support for the monophyly of the Teloschistales *s.l.*, whereas other studies placed the Lecanorales *s.str.*, sister to the Teloschistineae or Teloschistales *s.str.* (Fig. 2B), but without support and based on one or two ribosomal genes (e.g., Helms et al., 2003; Persoh et al., 2004; Wiklund and Wedin, 2003).

The high ML BP support for the Teloschistales sister to the Caliciales reported in our study or the monophyletic Teloschistales sensu Miadlikowska et al. (2006) (ML BP = 73% and 80%, respectively) was obtained only when restricting the analyses to taxa with the maximum number of loci (i.e., low number of taxa and low proportion of missing data). Also, in our study, this relationship was associated with a PP = 1 only when taxa with all genes (i.e., 45-taxon, 7-locus dataset) were included in the phylogenetic analysis. As we added taxa with missing sequences to the supermatrix, the resulting resolution of the three clades shifted from placing the Teloschistales sister to the Caliciales (Fig. 2A) to placing the Lecanorales sister to the Teloschistales (Fig. 2B and Table 3). This shift occurred more quickly with Bayesian analyses than with ML, with no support from the ML BP analyses for the latter relationship (Fig. 2B). However, with Bayesian analyses, the two alternative relationships received high support (Table 3). Conversely, the addition of taxa with missing sequences in Miadlikowska et al. (2006) constantly resulted in monophyly for their Teloschistales *s.l.*, with loss of ML BP, but with PP values >0.95 for that same relationship when all taxa were added to the supermatrix. However, contrary to our study, the number of taxa representing the Teloschistales *s.str.* and Caliciales remained low in the Miadlikowska et al. (2006) study, with 7 and 20 species, respectively, even when the 274 taxa representing all main lineages of the Lecanoromycetes were included in their supermatrix. Another difference between our study and the Miadlikowska et al. (2006) study was that they did not add taxa with fewer than 3 loci to their supermatrix.

We explored potential explanations for our inconsistent results (Fig. 2 and Table 3) by examining more closely the results from our single locus analyses. We found that the analyses of both amplicons of *RPB*2 recovered the Lecanorales as sister to the Teloschistales, whereas most ribosomal genes and *RPB*1 placed the Caliciales sister to the Teloschistales, but none of the single locus datasets provided ML bootstrap values ⩾70%. Facing the possibility of dealing with multiple evolutionary histories (gene trees), we intended to run a Bayesian concordance analysis with the program BUCKy (Ané et al., 2007; Larget et al., 2010) to estimate the concordance among gene trees. However, we confronted the problem of single gene Bayesian analyses not reaching convergence. Additionally, for genes with many missing taxa, as it was in our case, it is not clear if the typical number of sampled trees retained during the first stage of MCMC accurately samples the posterior distribution of tree topologies (Ané et al., 2007).

For this reason, we used the trees generated by the ML bootstrap analyses on each locus separately to determine the proportion of bootstrap trees per locus that recovered the two alternative relationships of interest (Table 4). As expected, bootstrap analyses of the nucSSU, nucLSU, and *RPB*1 generated a higher number of trees with the Caliciales sister to the Teloschistales, whereas the two amplicons of *RPB*2 and mitSSU generated a higher number of trees with Lecanorales as the closest relative to

Table 4
Number of ML bootstrap trees (out of 1000 replicates) recovering the two alternative sister relationships, i.e., the Caliciales sister to the Teloschistales (relationship 1, Fig. 2A) or the Lecanorales sister to the Teloschistales (relationship 2, Fig. 2B). The highest number of trees per locus is in bold.

Loci	Caliciales (1)	Lecanorales (2)
5.8S	0	0
mitSSU	1	**169**
nucLSU	**468**	191
nucSSU	**85**	0
*RPB*1 [A–D]	**194**	26
*RPB*2 [5–7]	68	**657**
*RPB*2 [7–11]	9	**244**

Teloschistales (Table 4). When combining the two *RPB*2 loci we obtained an ML BP = 75% for the Teloschistales + Lecanorales clade. This is the highest bootstrap support value this relationship ever received. Adding the mitSSU locus to the two-locus *RPB*2 data matrix resulted in a drop of ML BP value to 59% for that same relationship (Fig. 2B). When combining nucLSU, with nucSSU and *RPB*1 we recovered an ML BP = 84% for the Teloschistales + Caliciales clade (Fig. 2A). Therefore, the addition of taxa with missing data enables the signal from *RPB*2 to take over the signal from *RPB*1, nucSSU, and nucLSU, and support the Lecanorales as sister to the Teloschistales, but only within a Bayesian framework.

One potential explanation is that priors inherent to Bayesian analyses might be more likely to be misleading when data matrices of loci with high resolving power have large proportions of missing data. According to Lemmon et al. (2009), one of the factors that can affect the magnitude and direction of bias in topological support in Bayesian analyses is the relative rates of evolution for genes with missing versus non-missing sites. Several authors disagree with these conclusions. Wiens and Morrill (2010) found no evidence that extensive missing data can lead to misleading estimates of Bayesian phylogeny or support values. Instead, Wiens and Morrill (2010) point out that it is more a matter of sampling enough informative characters overall (Wiens, 2003). In our study, we happen to have enough informative characters, but maybe the distribution of the missing data through the added characters can have an effect, since most missing data come from protein-coding loci. On the other hand, Ekman and Blaalid (2011) suggested that the results by Lemmon et al. (2009) could be explained by the use of fixed and equal branch lengths across the tree, but with a default exponential branch length prior. In our study, we used the default branch-length prior as well, which seems to be relevant to tree length estimates, perhaps particularly so when including abundant missing data (Ekman and Blaalid, 2011). Several studies have called attention to problems of tree length overestimation in Bayesian analyses and the importance of assessing appropriate branch-length priors. Such priors seem to be more influential in partitioned analyses with parameter-rich models and partition-specific rate multipliers (Brown et al., 2010; Ekman and Blaalid, 2011; Marshall, 2010; Marshall et al., 2006). However, assessing the influence of branch-length priors was outside the scope of this study and the effect of the long-tree problem on topological inference (our main concern here) is still unclear.

Another putative explanation is that loci used in this study may have different evolutionary histories or the three clades involved (i.e., Teloschistales, Caliciales, and Lecanorales) diverged within a very short period of time, so that by chance a set of characters from one gene, or combination of genes, would slightly favor one relationship over another, especially when at least one of the clades is species rich, rendering their stem branch very short. Based on our results, the latter seems to be the best explanation, because a very short internode is resolving the relationships between these three clades (Fig. 1), and the Lecanorales (which is among the most species rich order of all Fungi) has a very short supporting internode (stem branch). Missing data may have a stronger effect on portions of the tree with short internodes than with long internodes. The two *RPB*2 amplicons are the loci with the largest amount of missing data when combined (Table 2). Moreover, current implementations of Bayesian phylogenetic methods can generate high posterior probabilities for the wrong relationships when involving short internodes (Alfaro et al., 2003; Lewis et al., 2005).

Close relationships between members of the Caliciales and Teloschistales have been reported in previous classifications that were based on phenotypic traits. For instance, Poelt (1973) defined a suborder Buelliineae (including the Candelariaceae, the Physciaceae, and the Teloschistaceae). Apart from the Candelariaceae that have unicellular ascospores and are now considered as a distinct order outside the Lecanoromycetideae (Wedin et al., 2005; Miadlikowska et al., 2006), some of the characters used by Poelt (1973) are still valid in supporting the sister relationship of the Caliciales with the Teloschistales. Most representatives of both orders are characterized by polar-diblastic ascospores, which frequently exhibit conspicuous endospore thickenings, and in some cases by pluriseptate ascospores (e.g., *Brigantiaea*, some *Buellia*). Furthermore, other features could point towards a common origin, after the earlier divergence of the Lecanorales. For example, Sipman (1983) questioned whether septal pores in the Megalosporaceae were real or merely thinner parts of septa (pseudopores), as frequently observed in the Physciaceae (Poelt and Mayrhofer, 1979). Also, members of the Physciaceae and Megalosporaceae have similar conidiophores (Vobis, 1980).

Apart from the inconsistent resolution of the relationships among Teloschistales, Caliciales, and Lecanorales, relationships within these clades were otherwise recovered without any inconsistency among our datasets. More protein-coding genes and data matrices with less missing data are needed to resolve the relationships among these three orders. Although the current classification (Hibbett et al., 2007; Lumbsch and Huhndorf, 2010; Kirk et al., 2008) accepts a monophyletic order Teloschistales sensu Miadlikowska et al. (2006), in this study we propose to elevate its two suborders Teloschistineae and Physciineae to the order level, Teloschistales and Caliciales, respectively. This re-classification changes the use of the name Teloschistales from an unstable node to a stable node. The order rank is widely used in mycology (much more than suborder), and it should always be more appropriate for consistently well-supported clades, rather than for unstable clades characterized by short, conflicting internodes. As well as resulting in a more stable classification in terms of genotypic evidence (that is, incidentally, independent of whatever eventual resolution is found for the relationship between the three orders), this change is also supported by phenotypic evidence (see Section 4.2).

4.2. Implications for the evolution of phenotypic traits and classification of the Teloschistales and Caliciales

4.2.1. Brigantiaeaceae sister to Letrouitiaceae–Letrouitineae

Our results confirm the classification of the Letrouitiaceae, Megalosporaceae, and Teloschistaceae within the Teloschistales. We report here the addition of the Brigantiaeaceae to this order, a family considered so far as *incertae sedis* in the subclass Lecanoromycetidae (Lumbsch and Huhndorf, 2010) or as a member of the Lecanorales (Kirk et al., 2008). Regardless of the apparent similarity between *Brigantiaea* and *Letrouitia*, the family Brigantiaeaceae had never been included within the Teloschistales due to differences in the ascospore and ascus characteristics (Hafellner and Bellemère, 1981b), except for Poelt (1973) who accepted *Brigantiaea* as a separate genus in the family Teloschistaceae. According to Hafellner and Bellemère (1981c), muriform ascospores and the production of anthraquinones in both genera were analogous character states. However, the sister relationship of *Brigantiaea* and *Letrouitia* resulting from our molecular phylogenetic study supports these traits as being homologous.

In general, the type of apothecia, muriform ascospores, regardless of their ontogeny, conidiophores, and conidia (Vobis, 1980) could represent potential synapomorphies in agreement with the sister relationship of the Brigantiaeaceae and Letrouitiaceae. Different secondary compounds in the thallus, ascus types (*Brigantiaea*-type versus *Letrouitia*-type; Hafellner and Bellemère, 1981b,c; Honegger, 1978), ascospore ontogeny, different types of pycnidia (*Umbilicaria*-type in *Brigantiaea* versus chambered in *Letrouitia*; Vobis, 1980), among other characters, justify maintaining them as separate families. Additionally, both the Brigantiaeaceae

and Letrouitiaceae are composed of mostly epiphytic species, and in both cases, most species have tropical and subtropical distributions. According to Hafellner (1997) the center of diversity of *Brigantiaea* is clearly circumpacific where more than 50% of all known species occur. For *Letrouitia*, most species are found in South Asia and Australia, including New Hebrides and New Caledonia (Hafellner, 1983). Such similar ecological preferences, patterns of distribution, and centers of diversity, suggest a common origin of these families. Most *Brigantiaea* species included in our study are from the Indo-Pacific region, except for *B. fuscolutea* that was collected in Alaska and has a bipolar distribution (Hafellner, 1997), and *B. sorediata* that was from Japan (Kashiwadani et al., 2002). Both species are derived from the first two divergences in the family and seem to be more distantly related to the remaining members of this genus. In our study we also included two species identified as *B.* cf. *leucoxantha* and *B.* cf. *tricolor*. They bear some similarities with *B. leucoxantha* and *B. tricolor*, but did not completely fit their description. According to Sipman (pers. comm.), these specimens might belong to undescribed species.

4.2.2. Megalosporaceae sister to Teloschistaceae–Teloschistineae

According to Sipman (1983), the attribution of the family Megalosporaceae to the Lecanorales seemed well supported by the ontogeny and structure of its ascomata, and fit well within the phenotypic variation described for this order by Henssen and Jahns (1974). However, molecular phylogenies revealed the Megalosporaceae sharing a most recent common ancestor with the Teloschistaceae (Lutzoni et al., 2004; Miadlikowska et al., 2006). In this study, we confirm this result with a much broader taxon sampling.

When compared to the Brigantiaeaceae and Letrouitiaceae, the Megalosporaceae share a similar thallus habit, type of apothecia, ascospores and paraphyses, but differs from these two families by their ascus type (*Megalospora*-type; Hafellner, 1984; Hafellner and Bellemère, 1981a) and their secondary compounds and lack of anthraquinones in their apothecial disks. The Teloschistaceae and Megalosporaceae have in common a similar ascospore ontogeny and structure, as well as septum formation. The fact that members of the Megalosporaceae lack anthraquinones, suggests a loss of these secondary compounds within the Teloschistales.

The segregation of the genus *Megaloblastenia* from the other two genera within the Megalosporaceae, based on ascospore structure (Sipman, 1983), seems well founded. To a certain extent, *Megaloblastenia* ascospores are reminiscent of the polarilocular ascospore type of the Teloschistaceae, supporting Hafellner's (1984) doubts that this genus belonged to the family Megalosporaceae.

The neotropical genus *Sipmaniella*, with a single species *S. sulphureofusca*, derived from the first divergence within the Megalosporaceae in our tree, resembles certain *Megalospora* species in several morphological features. Recently, Kalb et al. (2009) transferred *Lecania sulphureofusca* to a new genus *Sipmaniella*. Based on our results, *S. sulphureofusca* is placed within the Megalosporaceae rather than within the Lecanoraceae (Kalb et al., 2009; Lumbsch and Huhndorf, 2010). Its placement in the tree, as a first split within the Megalosporaceae, its high phylogenetic distance from the other two genera within this family, and phenotypic differences support its recognition as a distinct genus within this family.

As in the Brigantiaeaceae and Letrouitiaceae, species belonging to the Megalosporaceae are mostly epiphytic with a center of diversity in Australasia, but with representatives throughout the tropical and warm-temperate zones of the world. Sipman (1983) suggested the evolution of the family might have taken place on Gondwana-land, from which main migration routes would have brought species into tropical Africa and America, tropical eastern Asia, as well as southern South America.

Four different ascus types occur within the Teloschistales. Although the limited power of this character to predict relationships at high taxonomical levels has already been shown (Ekman et al., 2008), this trait might be useful to delimit families within the Teloschistales. Accordingly, the redefined Teloschistaceae (Poelt and Hafellner, 1980) consisting only of species with *Teloschistes*-type asci would be justified.

Conversely to the three other Teloschistales families, members of the Teloschistaceae have a mainly temperate distribution in both hemispheres, and are mostly saxicolous. Within the clade encompassing *Teloschistes s.str.* (including the genus type *T. flavicans*), referred to here as the 'Teloschistoid' clade, the predominance of fruticose taxa from mostly arid regions is noteworthy. Sister to this fruticoid group is a clade characterized mainly by lobed orange species of *Caloplaca*, *Xanthomendoza*, and *Xanthoria*, already revealed by previous studies (Arup and Grube, 1999; Gaya et al., 2003, 2008; Søchting and Lutzoni, 2003) and referred to therein as the 'Xanthorioid' clade. Within this clade, the genus *Xanthomendoza* has its center of diversity in Western North America, with only a limited number of species from Australasia and South America, and *X. borealis* considered as a true bipolar species by Lindblom and Søchting (2008). With only one locus obtained from GenBank, the genus *Josefpoeltia*, restricted to South America, is recovered within the core of the genus *Xanthomendoza* with high support at multiple internodes. The type species for both genera (*X. mendozae* and *J. parva*) are found within this clade. If this phylogenetic placement is confirmed in further studies including more loci for *Josefpoeltia*, *J. parva* (and potentially the other species of this genus) would be subsumed within *Xanthomendoza*.

In 2003, Kondratyuk and Kärnefelt described three new genera within the Teloschistaceae: *Oxneria*, *Rusavskia*, and *Xanthoanaptychia*, respectively segregated from *Xanthomendoza*, *Xanthoria*, and *Teloschistes*. These new genera have not been integrated in the most current classification of the Ascomycota (Myconet; Lumbsch and Huhndorf, 2010) because of a lack of molecular evidence supporting the taxonomic status proposed by Kondratyuk and Kärnefelt (2003). Our current results confirm that the morphological traits used to circumscribe those genera are not indicative of evolutionary relationships. The genus *Oxneria* (represented here by *Xanthomendoza fallax*, *X. hasseana*, *X. ulophyllodes*, and *X. weberi*) is polyphyletic and embedded within *Xanthomendoza. Rusavskia* (represented by *Xanthoria elegans*, *X. resendei*, and *X. sorediata*) is also polyphyletic but nested within a well-supported *Caloplaca–Xanthoria* clade, sister to *Xanthomendoza*. *Xanthoanaptychia* is a superfluous name for *Seirophora* (represented here by *S. contortuplicata* and *S. lacunosa*). Similar results were recently obtained by Fedorenko et al. (2009), albeit these authors disregarded the phylogenetic evidence, subscribing to the newly described genera.

Within the *Caloplaca–Xanthoria* clade, after the first divergence of *C. ochracea* and *C. squamosa s.l.*, the splits of the lineages represented by xanthorioid taxa from the Southern Hemisphere (e.g., *Xanthomendoza novozelandica*, *Xanthoria karrooensis*, *X. ligulata*), are mainly from Australasia and South Africa, suggesting that further taxon sampling might unveil a geographical pattern.

Fedorenko et al. (2009) recently described a new genus, *Jackelixia*, to encompass several Australasian species, among them *J. ligulata*, included in our study. Due to their non-conclusive phylogeny and unconvincing taxonomical decisions, we prefer maintaining this taxon in *Xanthoria* for now. Just as for *X. ligulata*, *X. karrooensis* has been recently transferred to a new genus, namely *Xanthokarrooa* (Fedorenko et al., 2009). In our topology, *X. karrooensis* and *X. ligulata* form a monophyletic entity, sister to the remaining *Xanthoria* and placodioid *Caloplaca* species.

Xanthodactylon is a genus restricted to South Africa. With only one representative included in our study, *X. turbinatum*, the phylogenetic placement of this genus sister to the *Caloplaca–Xanthoria* clade needs to be confirmed before its taxonomy is reassessed.

The two main monophyletic groups within the Teloschistaceae just discussed are provisionally named here as 'Teloschistoid' clade and 'Xanthorioid' clade, forming the well-supported subfamily Xanthorioideae. The clade sister to this subfamily, also recovered here with confidence, is composed of phenotypically diverse species of *Caloplaca*, the genus *Fulgensia*, the species of *Teloschistes* transferred to the genus *Seirophora* (Frödén and Lassen, 2004), and *Ioplaca*. We tentatively labeled this clade as the subfamily Caloplacoideae. Described by Poelt (1977) as endemic to the Himalayas and currently with only one species, *Ioplaca* appears as an early divergence within the subfamily Caloplacoideae.

With several species of the polyphyletic genus *Caloplaca* spread all over the Caloplacoideae clade, relationships within this clade confirm the results from Gaya et al. (2008), but this time with support. Whereas a few *Caloplaca* species have anthraquinones in the thallus, most taxa from this genus falling within the Caloplacoideae lack these secondary compounds or display them only in their apothecia. The same is true for the genus *Seirophora*, which includes taxa without anthraquinones in the thallus, except for *S. contortuplicata* and *S. aurantiacus*, but in small amounts (Søchting and Frödén, 2002). The genus *Fulgensia*, without polarilocular ascospores, shows a slightly different anthraquinone composition compared to *Caloplaca* (Søchting, 1997, 2001). In terms of growth forms, members of the Caloplacoideae are mostly characterized by crustose taxa, with the exception of the fruticose *Seirophora*. In light of the currently available data, it seems that anthraquinones could have been partially lost within the Caloplacoideae.

Our molecular phylogenetic study confirms that the genus *Caloplaca* was defined based on symplesiomorphic characters (such as the crustose growth form and presence of anthraquinones) rendering the taxonomic disentanglement of the Teloschistaceae virtually impossible without a broad and comprehensive molecular phylogenetic survey. This situation is nearly identical to the one encountered in the Verrucariaceae (Gueidan et al., 2007, 2009) where the genus *Verrucaria* had been defined based also on symplesiomorphies. We are implementing for the Teloschistaceae the same global phylogenetic strategy that was used successfully to revise the classification of the Verrucariaceae. We hope that the recognition of new genera will be more stable and meaningful within the Teloschistaceae if this is done within a comprehensive and collaborative multi-locus phylogenetic framework.

4.2.3. Caliciales

The phenotypic characters that seem to best track the evolution of the Caliciales and Teloschistales and their families are ascospore shape and ontogeny, as well as ascus structure and secondary compound composition.

Within the Caliciales, according to Helms et al. (2003), ascus characters and hypothecium pigmentation could be supported as morphological synapomorphies for the delimitation of the two main lineages (i.e., *Buellia* and *Physcia* groups). The current circumscription of the Physciaceae (Lumbsch and Huhndorf, 2010) is equivalent to the Physciineae as proposed by Miadlikowska et al. (2006) and to the Caliciales as shown here (Fig. 1). The current classification of the Physciaceae does not take into consideration the well-supported phylogenetic structure within this group containing clades with distinctive phenotypes. Helms et al. (2003) addressed this problem formally accepting and emending the families Caliciaceae and Physciaceae, which corresponded to their clades B and A (i.e., *Buellia* and *Physcia*-groups), respectively, to encompass this phylogenetic structure. Miadlikowska et al. (2006) followed this proposition recognizing the two families within the Physciineae. Here we adopt this classification to reflect this initial split within the newly circumscribed Caliciales (Fig. 1). The first divergence within the Caliciaceae leads to a clade with typical members of this family, represented here by *Calicium viride* and *Cyphelium tigillare*, and to a clade of taxa centered around *Buellia s.l.* To easily refer to these two groups we propose provisionary subfamily names within the Caliciaceae – Calicioideae and Buellioideae.

Physciaceae and Caliciaceae as defined here (Fig. 1) have been characterized extensively, morphologically, and anatomically by Helms et al. (2003) and Nordin (2000). The close relationship of the Calicioideae and Buellioideae is reflected by the presence of a true excipulum, *Beltraminia*-type ascospores, and similar ascospore ontogeny and ornamentation (Helms et al., 2003; Nordin, 2000; Wedin et al., 2000b).

With the new subfamily circumscription proposed here (Calicioideae and Buellioideae), taxa considered part of the traditional family Caliciaceae *s.str.*, do not have a single origin. Two of the included species are recovered in the Calicioideae, whereas *Texosporium*, *Thelomma*, and *Tholurna* form a monophyletic group within the Buellioideae. This result is in disagreement with previous studies that recovered a closer relationship for the taxa mentioned above (Helms et al., 2003; Tibell, 2003; Wedin et al., 2000a, 2002). Only Miadlikowska et al. (2006) showed different origins for *Calicium* and *Tholurna*, but they never recovered the early diverging clade that we call here Calicioideae. Based on our results, the hypothesis that the evanescent asci, with passive spore dispersal, and the development of a mazaedium, are a secondary reduction from a nonprototunicate ascus with an apical dehiscence apparatus and active spore dispersal (Tibell, 2003; Wedin et al., 2000b, 2002) is confirmed, and has occurred more than once within the Caliciaceae. Prototunicate asci and mazaedia are present in many unrelated ascomycete groups and it is clear that these traits have evolved several times outside this family. With the current taxon sampling it is difficult to establish which phenotypic traits distinguish the subfamilies Buellioideae and Calicioideae. More taxa have to be included and a thorough phenotypic revision needs to be done to better understand the undergoing evolution of these two subfamilies.

5. Conclusions

These results highlight the difficulty of assessing monophyly for deep short internodes when a cumulative supermatrix approach with increasing amounts of missing data is used. Whereas the genes used so far have evidently improved the level of support and resolution throughout the newly circumscribed Caliciales and Teloschistales, their relationship needs yet to be confirmed with more genes and less missing data.

This study represents the first attempt to implement a global phylogenetic strategy to revise the classification of the Teloschistales with the recognition of several new suprageneric taxa. We hope this will form the foundation for a more stable and meaningful classification within this species rich order of lichen-forming fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2012.01.012.

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