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Disentangling the *Collema-Leptogium* complex through a molecular phylogenetic study of the Collemataceae (Peltigerales, lichen-forming Ascomycota)

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Abstract: Family Collemataceae (Peltigerales, Ascomycota) includes species of cyanolichens with foliose to fruticose or crustose thalli, with simple or septate ascospores. The current classification divides this family into two groups on the basis of ascospore types. The objective of this study was to evaluate the phylogenetic relationships within this family. Combined DNA sequence data from the nuclear large subunit and mitochondrial small subunit ribosomal RNA genes were used to evaluate monophyly of the family and the relationships between the largest genera of this family. The results revealed that this family is not monophyletic. Genera *Staurolemma* and *Physma*, currently classified within the Collemataceae, were found nested within the Pannariaceae. The second result of this study confirms that the genera *Collema* and *Leptogium*, both part of the Collemataceae *s. str.*, are not monophyletic and that the presence of a thallus cortex is not a synapomorphy for *Leptogium*. The main taxonomic conclusion is that families Collemataceae and Pannariaceae were recircumscribed in light of molecular findings with the latter family now including *Staurolemma* and *Physma*. Genera *Collema* and *Leptogium* form a single mixed monophyletic group. Inferred ancestral character states within the *Collema-Leptogium* complex revealed that the ancestor of this family had a thallus without cortex and that a cortex evolved at least twice relatively early in the evolution of the Collemataceae *s. str.* These independent gains of a thallus cortex seems to be associated with a transition from colonizing bare rocks and soils in semi-arid and exposed habitats to epiphytism in shady humid forests.

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INTRODUCTION

The cosmopolitan family Collemataceae includes fungal species forming lichen symbiotic associations with cyanobacteria (*Nostoc*). Thalli of these lichens range from crustose, foliose to fruticose, with a typical homiomerous thallus organization (occasionally parapletenchymatous throughout). The thallus can be eucorticate, pseudocorticate or noncorticated. No secondary substances typical of lichens have been reported for this family (Culberson 1969, Degelius 1954, Kirk et al. 2008). One of the first well developed taxonomical arrangements of this family was made by Zahlbruckner (1921–1930), who proposed 11 genera. Degelius (1954) included 13 genera. Finally, using ascocarp ontogeny as a grouping criterion, Henssen (1965) recognized seven genera within this family. Under the current classification (Lumbsch and Huhndorf 2007) the family comprises eight genera, *Collema* F.H. Wigg., *Homothecium* A. Massal., *Leciophysma* Th. Fr., *Leightoniella* Henssen, *Leptogium* (Ach.) Gray, *Physma* A. Massal., *Ramalodium* Nyl. and *Staurolemma* Körb. On the basis of ascospore morphology the Collemataceae has been divided into two groups (Degelius 1954, Sierk 1964, Henssen 1965, Jørgensen and Henssen 1999). The first group includes taxa with septate ascospores, that is *Collema* (ca. 80 species) and *Leptogium* (ca. 189 species) (Kirk et al. 2008). The members of the second group (composed of the remaining six genera) have simple ascospores. In contrast to the Collemataceae with septate ascospores the genera belonging to the group with simple ascospores include a small number of species (ca. 10 known species), most of which with restricted distribution.

The taxonomic and phylogenetic position of the Collemataceae is well established (Miadlikowska and Lutzoni 2004, Miadlikowska et al. 2006, Hofstetter et al. 2007), being part of suborder Collematineae within the Peltigerales (Lecanoromycetes). The Collematineae includes four families, Coccocarpiaceae, Collemataceae, Pannariaceae and Placynthiaceae (Miadlikowska et al. 2006, Lumbsch and Huhndorf 2007). Phylogenetic studies by Miadlikowska and Lutzoni (2004), Miadlikowska et al. (2006) and

Wiklund and Wedin (2003) supported the morphology-based monophyletic status of these families. However their conclusions were based on a limited sampling of the Collemataceae, including only the two largest genera of this family, *Collema* and *Leptogium*. The only family within the Collematineae that was relatively well represented in previous phylogenetic studies has been the Pannariaceae. A broad multilocus phylogenetic survey of the Lecanoromycetes by Miadlikowska et al. (2006), which included seven of the 17 genera classified within the Pannariaceae (Lumbsch and Huhndorf 2007), was in agreement with the Pannariaceae being monophyletic. This contradicted a study of the Pannariaceae by Ekman and Jørgensen (2002) that was based on the nuclear ribosomal internal transcribed region (nrITS) sequenced from seven genera within this family, after which they concluded that the family was not monophyletic. These results demonstrate the need for sequencing multiple independent loci for a more comprehensive representation of genera within the Collematineae, when testing the monophyletic status of families classified within this suborder.

Genera *Collema* and *Leptogium* are distinguished respectively by a thallus without or with a cortical layer (Degelius 1954, Sierk 1964, Kirk et al. 2008). Contrary to the members of the Collemataceae that have septate ascospores (*Collema* and *Leptogium*), the thallus cortex was not judged to be of importance when classifying genera of collemataceous lichens with simple ascospores. These genera are defined by two ascoma characteristics (Henssen 1981, Henssen 1999). First, the presence or absence of a thalline margin establishes two groups, genera *Homothecium*, *Leciophysma* and *Ramalodium* (without a thalline margin) and genera *Leightoniella*, *Physma* and *Staurolemma* (with this feature). Ascus structure was the second characteristic used to segregate genera within this group of simple ascospore collemataceous lichens (Henssen 1979, Jørgensen and Henssen 1999). Genera *Ramalodium* and *Staurolemma* have asci without apical amyloid structures, while *Homothecium*, *Leciophysma*, *Leightoniella* and *Physma* have asci with amyloid apical structures. Although genera with simple ascospores have asci and ascospores similar to some Pannariaceae genera, their inclusion within the Collemataceae have not been questioned so far.

Many phylogenetic studies have revealed that morphological, anatomical and chemical characters traditionally used to classify ascomycetous taxa were often poor predictors of monophyly. Likewise neglected characters or characters that were deemed less important were often better indicators of phylogenetic relationships (Miadlikowska and Lutzoni 2000, DePriest 2004, Schmitt and Lumbsch 2004, Divakar et al. 2005, Miller and Huhndorf 2005, Schmitt et al. 2005, Blanco et al. 2006, Divakar et al. 2006, Gueidan et al. 2007). The monophyletic status of morphology-based genera within the Collemataceae has not been assessed with a phylogenetic approach, except for *Collema* and *Leptogium*. Phylogenetic studies by Wiklund and Wedin (2003), Miadlikowska and Lutzoni (2004) and Miadlikowska et al. (2006) found that *Collema* and *Leptogium* are not monophyletic. Wiklund and Wedin (op cit.) also suggested that *Collema* is nested within *Leptogium*. However these molecular phylogenetic studies were designed to address phylogenetic relationships within the Peltigerales or the Lecanoromycetes, and therefore their sampling were minimal within genera such as *Collema* and *Leptogium*. Nevertheless the fact that *Collema* and *Leptogium* are not monophyletic genera suggests that phenotypic traits, other than the thallus cortex, need to be explored. Therefore it is necessary to investigate the phylogenetic relationships within this family to determine which traits support monophyletic groups in this family.

To evaluate the monophyly of the Collemataceae and its genera we analyzed partial sequence data from the nuclear large subunit and mitochondrial small subunit ribosomal RNA genes (nrLSU and mtSSU) of several species of Collemataceae representing the two main lineages of the family. Furthermore we studied the evolution of the thallus cortex with maximum likelihood and two Bayesian approaches. Our main goal was to clarify these questions: (i) Is the Collemataceae family a monophyletic entity? (ii) What is the phylogenetic value and significance of traditional morphological characters within the Collemataceae and closely related families? and (iii) What is the evolutionary pattern of the thallus cortex within the Collemataceae lineage?

MATERIALS AND METHODS

Taxon sampling.—Sequence data of the mtSSU and nrLSU were obtained from a total of 40 Collemataceae samples including one *Physma* and three *Staurolemma* specimens, as well as species representatives of the morphological, anatomical and ecological diversity within *Collema* and *Leptogium*. The geographic origin of the material, voucher specimens and GenBank accession numbers are provided (TABLE I). Specimens of genus *Ramalodium* (from herbaria) also were sampled, but it was not possible to sequence their DNA. Fresh material of *Homothecium* and *Leightoniella* could not be obtained for this study. To evaluate the monophyly of the Collemataceae, nrLSU and mtSSU sequences of eight species of Pannariaceae, Placynthiaceae and Nephromataceae were obtained from GenBank (TABLE I).

DNA sequencing.—DNA isolation, PCR amplification, PCR

TABLE I. List of taxa included in each of the datasets of this study, with country of origin, location of voucher, and GenBank accession number. Sequences obtained from GenBank are in bold

Species name	Dataset[a]	Origin/voucher[b]	GenBank accession number	
			mtSSU	nrLSU
Collema auriforme	1, 2	Spain, MA 16249	EU982568	EU982609
C. callopismum	1, 2	Spain, MA 13370	EU982572	EU982613
C. conglomeratum	2	Spain, MA 16238	EU982574	EU982615
C. cristatum	2	—	**DQ917409**	**DQ917408**
C. curtisporum	2	Spain, MACB 88658	EU982573	EU982614
C. flaccidum	2	Spain, MA 16253	EU982578	EU982618
C. fragrans	1, 2	Spain, MA 16239	EU982558	EU982599
C. fragrans	2	Spain, MA 16240	EU982575	EU982616
C. fragile	2	Spain, MA 16241	EU982576	EU982617
C. furfuraceum	1, 2	Spain, MA 16260	EU982567	EU982608
C. multipartitum	1, 2	Spain, MA 13393	EU982557	EU982598
C. nigrescens	1, 2	Spain, MA 16262	EU982563	EU982604
C. polycarpon	1, 2	Spain, MA 16264	EU982564	EU982605
C. tenax	2	Spain, MA 13396	EU982556	EU982597
C. tenax	2	Spain, MA 16269	EU982579	—
C. tenax	1, 2	Spain, MA 16268	EU982580	EU982619
C. undulatum	1, 2	Spain, MA 16036	EU982554	EU982595
Degelia plumbea	1	—	**AY340491**	**AY340543**
Fuscopannaria ignobilis	1	—	**DQ917416**	**DQ917417**
Leptogium arsenei	2	USA, DUKE 48101	EU982581	EU982620
L. austroamericanum	2	USA, DUKE 30733	EU982582	EU982621
L. brebissonii	2	Spain, MA 16275	EU982583	EU982622
L. burnetiae	2	Spain, MA 16242	EU982584	EU982623
L. corticola	2	Spain, MA 16278	EU982585	EU982624
L. cyanescens	2	Spain, MA 16279	EU982586	EU982625
L. cyanescens	1, 2	USA, DUKE 39467	EU982561	EU982602
L. furfuraceum	1, 2	Spain, MA 16280	EU982553	EU982594
L. lichenoides	1, 2	—	**DQ923120**	**DQ917412**
L. magnussonii	1, 2	Spain, MA 16288	EU982565	EU982606
L. microphylloides	2	Spain, Martínez 128-03 MA	EU982587	EU982626
L. phyllocarpum	2	Colombia, COL 509807	EU982588	EU982627
L. phyllocarpum	2	Costa Rica, DUKE 38734	EU982589	EU982628
L. pseudofurfuraceum	1, 2	Argentina, MA 16291	EU982562	EU982603
L. pulvinatum	2	Spain, MA 16032	EU982590	EU982629
L. saturninum	1, 2	France, MA 16024	EU982569	EU982610
L. schraderi	1, 2	Spain, MA 16243	EU982559	EU982600
L. subaridum	2	Spain, MA 16244	EU982591	EU982630
L. tenuissimum	1, 2	Spain, MA 16245	EU982552	EU982593
L. turgidum	2	Spain, MA 12868	EU982592	EU982631
Nephroma bellum	1	—	**AY300895**	**AY424211**
N. parile	1	—	**AY584625**	**AY340557**
N. resupinatum	1	—	**AY124169**	**AF286830**
Pannaria rubiginosa	1	—	**AY340513**	**AY340558**
Placinthyum nigrum	1	Spain, MA 10811	EU982566	EU982607
Protopannaria pezizoides	1	—	**AY340519**	**AY340561**
Physma byrsaeum	1	Vanuatu, CBG 99127161	EU982571	EU982612
Psoroma hypnorum	1	—	**AY340523**	**AY424210**
Staurolemma omphalarioides	1	Spain, MA 16247	EU982560	EU982601
S. omphalarioides	1	Spain, MA 16246	EU982555	EU982596
S. weberi	1	USA, ASU 237180	EU982570	EU982611

[a] 1 corresponds to the Collematineae dataset, 2 corresponds to the *Collema-Leptogium* dataset.

[b] Country of origin, with herbarium abbreviation and accession number, or with collector, collector number and herbarium abbreviation.

product purification, PCR sequencing reactions and automated sequencing were performed according to the methodology of Otálora et al. (2008). The nrLSU was amplified with the primer pair LR0R (Rehner and Samuels 1994)–LR7 (Vilgalys and Hester 1990). Because only a few samples could be amplified with this primer pair we designed the specific primer nrLSU 0170-5′ (5′-CCYTTC-GACGACTCGAGT T-3′) with FastPCR (Kalendar 2005), which was used in combination with LR7 on the remaining samples. For mtSSU the primer pair mtSSU1-mtSSU3R (Zoller et al. 1999) was used. The purified PCR products were sequenced with the same amplification primers, as well as LR6, LR2R and LR3R (Vilgalys and Hester 1990) for the nrLSU region.

Sequence alignments.—Sequences were aligned manually based on the secondary structure of *Saccharomyces cerevisiae* (Larsen et al. 1993) with MacClade 4.01 (Maddison and Maddison 2001). Ambiguously aligned regions were delimited following Lutzoni et al. (2000) and excluded from phylogenetic analyses. Two data matrices were assembled. The first taxon sampling (Collematineae dataset) was designed to evaluate the monophyly of the Collemataceae. This dataset included 17 *Collema* and *Leptogium* species, two *Staurolemma*, one *Physma* and nine species representing Pannariaceae, Placynthiaceae and Nephromataceae (TABLE I). Members of the Nephromataceae were selected as outgroup for the Collematineae ingroup based on phylogenetic studies of the Peltigerales (Wiklund and Wedin 2003, Miadlikowska and Lutzoni 2004). The main underlying goal of the second taxon sampling (*Collema-Leptogium* dataset) was to infer phylogenetic relationships within the Collemataceae. A total of 37 samples representing 14 *Collema* and 18 *Leptogium* species were selected. *Placynthium nigrum* was chosen as outgroup for this second dataset based on the phylogenetic analysis of the Collematineae dataset. The sequences alignments have been deposited in TreeBASE (accession numbers SN4540-22906, SN4540-22908).

Phylogenetic analyses.—Specific step matrices were obtained for each locus with StMatrix 4.2 (Lutzoni and Zoller, Duke University, www.Lutzonilab.net/downloads/). Weighted maximum parsimony analyses (wMP) of individual and combined nrLSU and mtSSU were performed for each of the datasets (Collematineae and *Collema-Leptogium*) with PAUP 4.0b10 (Swofford 2002). For each wMP analysis a heuristic search of 1000 random addition sequences (RAS) was conducted, with TBR branch-swapping, the MULTREE option was in effect and zero-length branches were collapsed. Bootstrap analyses (Felsenstein 1985) were used to estimate phylogenetic uncertainty with heuristic searches as described above on 1000 bootstrap datasets, with the exception that two (*Collema-Leptogium* dataset) and five (Collematineae dataset) RAS per bootstrap replicate were specified based on the high resolving power of the original data when 1000 RAS were implemented. The combinability of the single-locus datasets was assessed by visual inspection of the individual bootstrap values (Wiens 1998, Mason-Gamer and Kellogg 1996). Clades supported by bootstrap values ≥ 70% were compared between individual data partitions. A conflict was considered significant when one data partition supported a monophyletic group with bootstrap values ≥ 70% and the other data partition supported the same group as nonmonophyletic with bootstrap values ≥ 70%. Because no significant conflicts were detected it was assumed that the two datasets were congruent and could be combined in both cases (Collematineae and *Collema-Leptogium* datasets).

The combined datasets also were analyzed with a Bayesian approach. The Bayesian analyses were performed with a parallelized version of MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The optimal models of nucleotide substitution of nrLSU and mtSSU were selected for each data matrix with the Akaike information criterium (AIC) as implemented in Modeltest (Posada and Crandall 1998). For the combined Collematineae dataset the general time reversible model of nucleotide substitution (Rodriguez et al. 1990), including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR + I + G), was used for both nrLSU and mtSSU data partitions. For the second dataset (*Collema-Leptogium*) the same model also was selected for nrLSU, while a Hasegawa-Kishino-Yano model including estimation of invariant sites and assuming a discrete gamma distribution with two rate categories was selected for the mtSSU dataset. MrBayes software was run 5 000 000 generations on each combined dataset, starting from an initial random tree and employing four simultaneous chains. A tree was saved every 100th generation. The first 1000 saved trees for the Collematineae dataset were discarded as burn in. In the same way the first 800 saved trees were discarded for the *Collema-Leptogium* dataset. For the remaining trees in each analysis a majority rule consensus tree was assembled with the SUMT option of MrBayes, and posterior probabilities were calculated with the majority rule consensus tree command in PAUP*.

Character evolution (Collema-Leptogium).—Because it has been demonstrated that different methods produce different results with the same data (Ekman et al. 2008, Gueidan et al. 2008) we inferred ancestral states and traced the evolution of the thallus cortex for the *Collema-Leptogium* group by employing three methodologies with the last 8000 trees resulting from the combined Bayesian analysis. Maximum likelihood (ML) ancestral state reconstruction was performed with Mesquite 2.01 (Maddison and Maddison 2007) with the ML model MK1. Ancestral reconstruction with the Bayesian approach was carried out using SIMMAP v.1. Beta 2.3 (Bollback 2006). The option of MULTIPLE MAPPING with the number of realizations for each tree/each site set to 100 repetitions was carried out over three different morphological priors to test the influence on the results. SIMMAP implements overall transformation rate priors for multistate characters. The priors on the overall transformation rate were selected according to studies that used the same methodology (Glenner and Hebsgaard 2006, Gueidan et al. 2007, Ekman et al. 2008) as follows: (i) $\alpha = 3.0$, $\beta = 2.0$ and $k = 60$; (ii) $\alpha = 1$, $\beta = 1$ and $k = 60$; (iii) $\alpha = 5$, $\beta = 5$ and $k = 60$. Another Bayesian analysis was performed with software BayesTraits 1.0 (Pagel

et al. 2004), and two analyses were implemented using this program. First a REVERSIBLE-JUMP MCMC with a uniform hyperprior (0,10) was used on an unrestricted model with eight transformation rates. Second, a similar REVERSIBLE-JUMP MCMC was used but on a single-rate model.

RESULTS

Collematineae dataset.—The nrLSU data matrix contained 1145 unambiguously aligned sites of which 282 were variable and 220 parsimony informative. Eight ambiguously aligned regions were excluded (221 sites). The mtSSU data matrix consisted of 707 sites. A total of 137 sites, found in 12 ambiguously aligned regions, were excluded. Of the remaining sites 187 were parsimony informative.

Two equally most parsimonious trees were found, each 2257.91 steps long, based on the concatenated dataset. The strict consensus tree did not contradict the Bayesian tree topology. Fifteen internodes have bootstrap support $\geq$ 70%, and 19 have posterior probabilities $\geq$ 0.95 (FIG. 1). The results and phylogenetic information of mtSSU, nrLSU and combined datasets, based on parsimony and Bayesian analyses, are summarized (APPENDIX I, available online as supplementary material).

Family Collemataceae, including genera with simple ascospores, is not monophyletic. Genera *Staurolemma* and *Physma*, which traditionally are classified within the Collemataceae, are nested within the Pannariaceae clade, forming a strongly supported group. Both *Staurolemma omphalarioides* and *S. weberi* form a well supported clade, sister of *Pannaria rubiginosa*. The phylogenetic placement of *Physma byrsaeum* is unresolved within the Pannariaceae (FIG. 1).

Collema-Leptogium *complex dataset.*—The aligned concatenated data matrices consisted of 776 unambiguous nucleotide positions for mtSSU and 1302 for nrLSU after excluding 91 and 46 ambiguously aligned sites respectively. The number of parsimoniously informative characters was 164 for the mtSSU and 205 for nrLSU. The wMP analysis resulted in eight equally most parsimonious trees of 2042.90 steps. The majority rule consensus tree resulting from the Bayesian analysis was identical to the parsimony topologies for the well supported portions of the tree. The results and phylogenetic information of individual and combined datasets are summarized (APPENDIX I). The Bayesian phylogram with posterior probabilities and wMP bootstrap support values are shown (FIG. 2).

Both analyses revealed four main, well supported, monophyletic groups (FIG. 2; groups A–D). Group A is divided into two main well supported clades corresponding to the terricolous species *Leptogium turgidum* and *L. schraderi* and to a group composed mainly of epiphytic and facultative substrate species of *Leptogium* and *Collema*. Group A includes *Leptogium* species of sections *Collemodium*, *Leptogium* and *Homodium* as well as *Collema* species classified in the *tenax* and *fragrans* groups. Species of the *Homodium* section form a well supported monophyletic group (*L. tenuissimum*, *L. subaridum* and *L. magnussonii*), which is a sister group of the *Collemodium-fragrans-tenax* clade. Group B is formed by *Collema* species currently classified within the *leptogioides*, *cristatum* and *tenax* groups, which only include terricolous and saxicolous species. Group C is composed mainly of epiphytic *Collema* and *Leptogium* species forming four distinct clades (FIG. 2). Two of these clades include *Leptogium* species of section *Mallotium*. Another clade includes four *Collema* species of the *nigrescens* and *flaccidum* groups characterized by transversally septate ascospores, while the fourth clade exclusively includes species of the *Leptogium* section, including the species of the complexes *azureum*, *phyllocarpum* and *chloromelum*. Group D includes terricolous and saxicolous species of the *tenax* group. The sister relationship of clades C and D is well supported only by wMP bootstrap. The phylogenic placement of the saxicolous and terricolous *Collema* species of the monospecific *multipartitum* and *callopismum* groups (Degelius 1954) remains uncertain (FIG. 2).

Character evolution within the Collema-Leptogium *complex.*—Ancestral character states were inferred by three methods (TABLE II) for eight statistically significant nodes within the *Collema-Leptogium* group (FIG. 2). The hierarchical Bayesian reconstructions of the ancestral character states with SIMMAP and BayesTraits did not show any significant differences when using different morphological priors (SIMMAP) and models (BayesTraits); therefore only one of the datasets is shown for each of the analyses (TABLE II). The results from the three methodologies support the lack of a thallus cortex for the ancestor of the *Collema-Leptogium* clade. Based on results (TABLE II), a thallus with cortex evolved at least two times independently (nodes 3 and 7) followed by at least two losses of this feature (on the internode connecting nodes 7 and 6, and in group A) during the evolutionary history of *Collema-Leptogium* lichens.

DISCUSSION

The current inclusion of genera within the Collemataceae is based on phenotypic features of the thallus, together with ontogenetic characteristics of the ascoma (Degelius 1954, Sierk 1964, Henssen 1965,

FIG. 1. Bayesian phylogram of the Collematineae showing a polyphyletic family Collemataceae based on combined nrLSU and mtSSU sequences. Thick branches represent posterior probabilities ≥ 0.95. Numbers above branches are bootstrap values obtained with wMP. Classification of genera corresponds to Lumbsch and Huhndorf (2007). To the right of the tree morphological characters are indicated next to each species names as follows: (1) Ascospore type: ▲= septate, △ = simple; (2) Ascal apex structure: ● = tube like structure, ○ = absent, ◎ = Peltigera type; and (3) Perispore/epispore: ✱ = present ✳ = absence.

Miadlikowska and Lutzoni 2004). However there are several differences in ascoma, ascus and ascospore characteristics among the genera in this family (Henssen 1965, Jørgensen and Henssen 1999). As mentioned above the family has been divided into two groups based on spore types (simple or septate, TABLE III). Moreover *Collema*, *Leptogium*, *Staurolemma*, *Leightoniella* and *Physma* have ascomata with thalline margins while *Leciophysma*, *Homothecium* and *Ramalodium* lack this feature. *Collema*, *Homothecium*, *Leptogium*, *Leciophysma*, *Leightoniella* and *Physma* have asci with amyloid apical structures, but asci of *Ramalodium* and *Staurolemma* do not have apical structures detectable with Lugol's solution. These

differences have not been taken into account to recircumscribe genera within families of the Collematineae, possibly due to different interpretations of their taxonomic importance (Hafellner 1984, Bellemère 1994, Ekman and Jørgensen 2002, Miller and Huhndorf 2005, Schmitt et al. 2005). However our results suggest that ascospore characteristics are a good predictor of phylogeny within Collematineae (FIG. 1).

Staurolemma *and* Physma *as members of the Pannariaceae.*—Our phylogenetic study of the Collematineae shows that family Collemataceae under the current classification is not monophyletic when including *Physma* and *Staurolemma*, which are two of the five small genera with simple ascospores within the Collemataceae (FIG. 1). *Staurolemma* and *Physma* have a homiomerous thallus that contains no detectable lichen secondary metabolites; these and other morphological features make them more similar to *Collema* and *Leptogium* species than to *Pannaria*. However *Staurolemma* and *Physma* are more closely related to the Pannariaceae (FIG. 1). This phylogenetic relationship is correlated with anatomical characters: (i) *Staurolemma* species have the same apical ascus structures that are found in the *Pannaria* species; and (ii) *Staurolemma*, *Physma* and all Pannariaceae genera have the same ascospore type, that is simple ascospores with ornamented walls, while *Collema* and *Leptogium* have septate ascospores with smooth walls (FIG. 1, TABLE III). Based on these features and our phylogenetic results, we expect the rest of Collemataceae genera with simple ascospores to be classified within the Pannariaceae instead of in the Collemataceae. However some species of the Placynthiaceae, which is the sister family of the Collemataceae *s. str.* (FIG. 1), have multiple septate ascospores while other have simple ascospores. Furthermore Placynthiaceae species share the same ascus structure with species of *Physma* and some Pannariaceae genera (i.e. *Degelia*, *Psoroma* and *Fuscopannaria*) and apothecia of genera *Placynthium*, *Homothecium*, *Ramalodium* and *Leciophysma* lack a thalline margin. Therefore the classification of *Ramalodium*, *Homothecium*, *Leciophysma* and *Leightoniella* needs to be based on a molecular phylogenetic study, which also is essential to determine the taxonomic importance of these and other traits for the Collematineae. Based on our results, we recircumscribe the Collemataceae and Pannariaceae with the latter family now including *Physma* and *Staurolemma*.

Collema *and* Leptogium.—These genera form a single mixed clade confirming phylogenetic studies of Wiklund and Wedin (2003), Miadlikowska and Lutzoni (2004) and Miadlikowska et al. (2006). The current genus delimitation of *Collema* and *Leptogium* is based on the presence/absence of a cortical layer, but this character is homoplasious and a poor predictor of genetic relationships within family Collemataceae (FIG. 2). Similarly current subgeneric classifications most often do not circumscribe monophyletic groups (FIG. 2).

Zalhbruckner's arrangement (1921–1930) of genus *Leptogium* was based on cortex characteristics, thallus anatomy and presence/absence of a tomentum. Only section *Homodium* seems to be monophyletic (FIG. 2), which corresponds to species with a crustose to minutely foliose thallus, which is parapletenchymatous throughout. Section *Leptogium* includes the largest number of species. It is characterized by the absence of a tomentum, nonparapletenchymatous thallus (homiomerous medulla of loosely interwoven hyphae) and the presence of an upper and lower cortex. Species of this section are scattered within clades A and C (FIG. 2). Species belonging to section *Mallotium* have thalli with a tomentum on the lower surface. A lack of phylogenetic resolution prevents us from concluding whether this section is monophyletic (FIG. 2).

Genus *Collema* was subdivided into 22 groups by Degelius (1974; 10 of which are monospecific) using ascospore, excipulum propium and thallus anatonomical traits. Eight of these 22 groups are represented in our sampling. The *tenax* group, which corresponds to species with euthyplectenchymatous excipulum propium and plicate lobes, is polyphyletic, with species falling in clades A, B and D (FIG. 2). *Collema conglomeratum*, which is an epiphytic lichen, belongs to clade A, while the terricolous and saxicolous *C. polycarpon* and *C. tenax* belong respectively to clades B and D. Species of the *cristatum* group (*C. cristatum*, *C. undulatum* and *C. auriforme*), characterized by undulated lobules, an euparaplectenchymatous excipulum propium, submuriform ascospores and known to colonize soils and rocks, are found within group B but do not form a monophyletic group. Representative species of this group share a most recent common ancestor with *Collema fragile* (*leptogioides* group, with subparaplectenchymatous excipulum propium and muriform ascospores) and *C. polycarpon* (*tenax* group). *C. fragrans* (the only representative of the *fragrans* group), which is morphologically similar to species of the *cristatum* group, but epiphytic, falls in clade A. However species of the *nigrescens* and *flaccidum* groups form a well supported monophyletic group nested within clade C (together with species of genus *Leptogium*). These two last *Collema* groups are morphologically and anatomically similar; they share the same ascospore shape and size, but the *flaccidum* group differs by having a

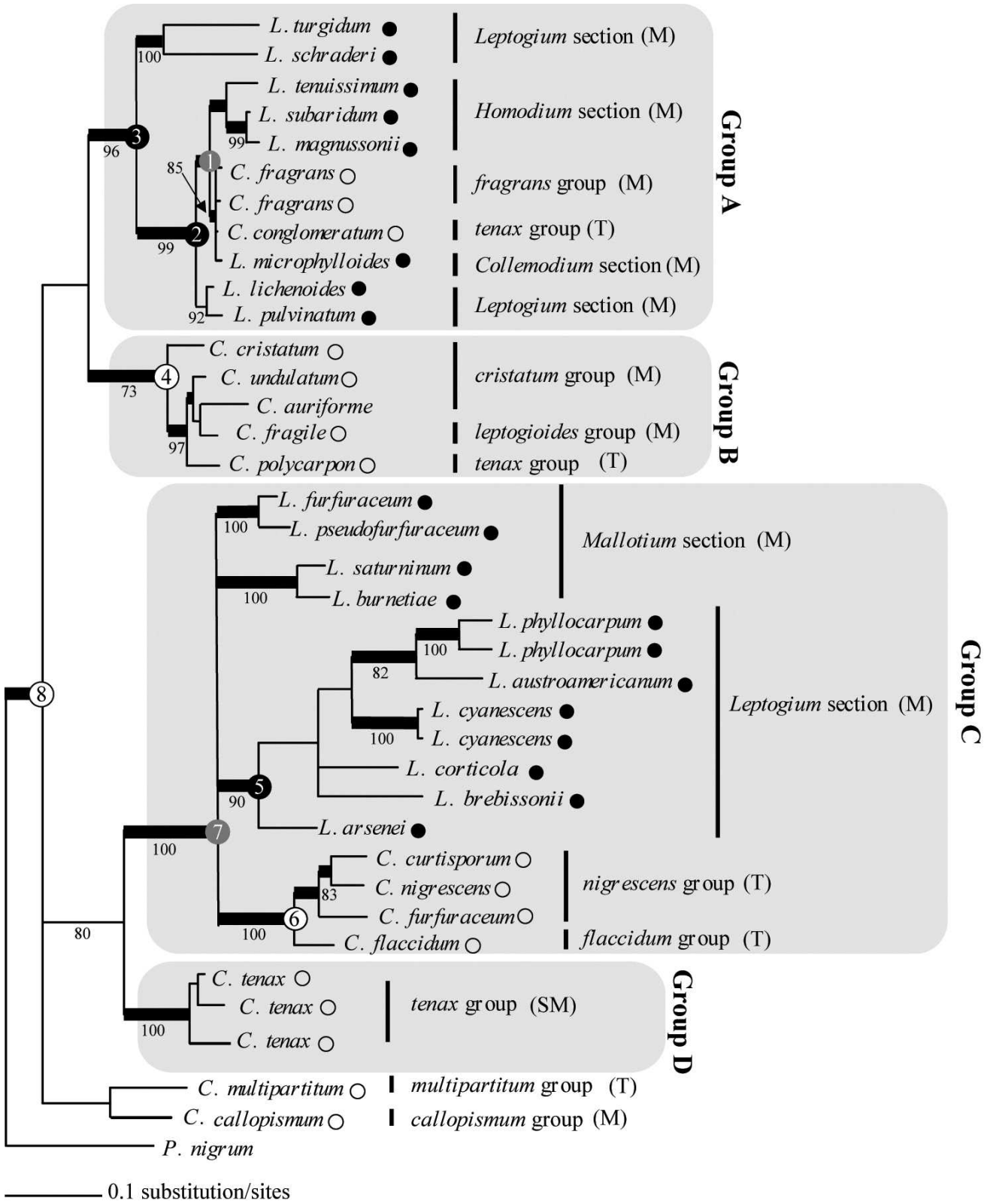

FIG. 2. Phylogenetic relationships among *Collema* and *Leptogium* species. The phylogram is a 50% majority rule consensus tree from the Bayesian analysis of the combined *Collema-Leptogium* data matrix. Internodes with posterior probabilities ≥ 0.95 are shown as thicker branches. Numbers above branches represent bootstrap values obtained with wMP. The numbered nodes represent those for which ancestral states were inferred by three methods (see TABLE II): ○ at an internal node = thallus

TABLE II. Ancestral state posterior probabilities for the presence/absence of a thallus cortex at eight nodes (FIG. 2) reconstructed with Mesquite, SIMMAP and BayesTraits

		Mesquite		SIMMAP		BayesTraits	
Node	State	cortex absence	cortex presence	cortex absence	cortex presence	cortex absence	cortex presence
1		0.012	0.655	0.264	0.737	0.123	0.877
2		0.000	0.788	0.033	**0.967**	0.025	**0.975**
3		0.000	0.501	0.094	0.906	0.041	**0.959**
4		**0.955**	0.000	**0.999**	0.001	**0.984**	0.016
5		0.000	**0.999**	0.001	**0.999**	0.026	**0.974**
6		**1.000**	0.000	**0.998**	0.002	**0.992**	0.008
7		0.000	0.464	0.138	0.863	0.090	0.910
8		**0.957**	0.000	**0.953**	0.047	0.886	0.114

smooth thallus, while species of the *nigrescens* group have a distinctly ridged thallus.

Ascospore septation does not seem to be a good predictor of phylogenetic relationships within the *Collema-Leptogium* complex because *Collema* species having ascospores without longitudinal septa (e.g. *Collema conglomeratum* and *C. polycarpom*) often are nested within *Collema* and *Leptogium* monophyletic groups with muriform ascospores (FIG. 2). Also the excipulum propium and thallus anatomy, which are other features used by Degelius to delimit artificial groups within *Collema*, are not synapomorphic traits because species sharing these character states fall in different clades.

Although clades A, B, C and D are well supported groups we have not found morphological or ecological traits supporting them. Because there are no obvious way to break down the *Collema-Leptogium* complex into few phenotypically or ecologically recognizable genera one solution would be to treat this complex as one genus, *Collema*, because this name has priority over *Leptogium*. The alternative strategy, consisting of describing many small genera within this complex, seems taxonomically counterproductive at this time and likely to lead to more confusion. Therefore although the main groups of these two genera, including the type species (*C. nigrescens* and *L. lichenoides*), were part of our study we think that proposing a new classification for genera *Collema* and *Leptogium* more in-depth phylogenetic, taxonomical and nomenclatural studies, including more members of each subgeneric entity, are needed.

Character evolution within the Collema-Leptogium *complex.*—The ancestral character state inferred at node 8 provides well supported evidence that the ancestor of the *Collema-Leptogium* complex was a lichen with a noncorticated thallus (FIG. 2, TABLE II). The transition to the corticated state occurred at least two times. Based on our sampling, one gain of the thallus cortex took place during the evolution of the lineage leading to group A (i.e. node 3) the other during the evolution of the lineage leading to group C (i.e. node 7, but at lower probabilities). Each gain was followed by at least one reversal to the noncorticated state (FIG. 2, TABLE II). In spite of a lack of consensus with respect to the adaptive value and functions of thallus cortex (Grube and Hawksworth 2007), our results indicated that the evolution of a thallus cortex within the *Collema-Leptogium* complex seems to be associated with transitions to a new substrate and habitat. Although both genera comprise saxicolous and terricolous species, *Collema* species with ancestrally ecorticated thalli (i.e. those belonging to groups B and D) tend to be larger and more frequent in semi-arid environments and exposed microhabitats (bare rocks and soil) than *Leptogium* species (corticated). The absence of a cortical layer is a morphological adaptation that allows thalli to uptake water rapidly (Rundel 1982), but it also increases the rate of water loss by

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cortex absent based on at least one posterior probability ≥ 95%; ● at an internal node = thallus with two cortices based on at least one posterior probability ≥ 95%; and ● at an internal node = thallus most likely with two cortices but without any posterior probability ≥ 95%. Open and full circles to the right of the species names represent observed states for extant species, where ○ = thallus cortex absent and ● = thallus with two cortices. To the right of the tree the classifications of *Collema* groups correspond to Degelius (1954, 1974) and the *Leptogium* sections to Sierk (1964). Letters in parentheses refer to ascospores septation: (M) = muriform ascospores, (SM) = submuriform ascospores, and (T) = ascospores with only transverse septa.

TABLE III. Comparison between ascospore characteristics of Collemataceae *s. lat.* genera and some Pannariaceae genera

Genus	Ascopore character		
	Ascospore type	Ascospore wall	Shape
Collemataceae *s. lat.*			
Collema	Septate, 1 to multiple septa, transversally septate/muriform	Smooth	Ellipsoid/bacilliform/globose/fusiform
Homothecium	Simple	Epispore	Subglobose/ellipsoid
Leciophysma	Simple	Perispore	Subglobose/ellipsoid
Leightoniella	Simple	Epispore	Ellipsoid
Leptogium	Septate, transversally septate/muriform	Smooth	Ellipsoid/subfusiform
Physma	Simple	Perispore	Ellipsoid
Ramalodium	Simple	Epispore	Ellipsoid
Staurolemma	Simple	Perispore	Globose /subglobose
Pannariaceae			
Degelia	Simple	Epispore	Ellipsoid
Fuscopannaria	Simple	Epispore	Ellipsoid
Pannaria	Simple	Perispore	Ellipsoid
Protopannaria	Simple	Epispore	Ellipsoid
Psoroma	Simple	Epispore	Ellipsoid

evaporation (Rundel 1982, Souza-Egipsy et al. 2000). Therefore thalli that lack a cortex, as *Collema* species do, need to increase their size to maximize water absorption in exposed habitats where humidity and dew are the primary water sources (Larson 1979, Rundel 1982). On the other hand the gain of a thallus cortex is related with foliose species inhabiting mainly old shady forests (groups A and C). However these assumptions must be confirmed through further molecular and physiological studies.

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LITERATURE CITED

Bellemère A. 1994. Asci and ascospores in ascomycete systematics. In: Hawksworth DL, ed. Ascomycete systematics problems and perspectives in the Nineties. NATO Advanced Science Institutes Series. New York: Plenum Press. p 111–127.

Blanco O, Crespo A, Ree RH, Lumbsch HT. 2006. Major clades of parmelioid lichens (Parmeliaceae, Ascomycota) and the evolution of their morphological and chemical diversity. Mol Phylogenet Evol 39:52–69.

Bollback JP. 2006. SIMMAP: stochastic character mapping of discrete traits on phylogenies. BMC Bioinformatics 7:88.

Culberson CF. 1969. Chemical and botanical guide to lichen products. Chapel Hill: Univ North Carolina Press.

Degelius G. 1954. The lichen genus *Collema* in Europe: morphology, taxonomy, rcology. Symb Bot Upsal 13:1–499.

———. 1974. The lichen genus *Collema* with special reference to the extra-European species. Symb Bot Upsal 20:1–215.

DePriest PT. 2004. Early molecular investigations of lichen-forming symbionts: 1986–2001. Annu Rev Microbiol 58: 273–301.

Divakar PK, Crespo A, Blanco O, Lumbsch HT. 2006. Phylogenetic significance of morphological characters in the tropical *Hypotrachyna* clade of parmelioid lichens (Parmeliaceae, Ascomycota). Mol Phylogenet Evol 40:448–458.

———, Molina MC, Lumbsch HT, Crespo A. 2005. *Parmelia barrenoae*, a new lichen species related to *Parmelia sulcata* (Parmeliaceae) based on molecular and morphological data. Lichenologist 37:37–46.

Ekman S, Andersen HL, Wedin M. 2008. The limitations of ancestral state reconstruction and the evolution of the ascus in the Lecanorales (lichenized Ascomycota). Syst Biol 57:141–156.

———, Jørgensen PM. 2002. Toward a molecular phylogeny for the lichen family Pannariaceae (Lecanorales, Ascomycota). Can J Bot 80:625–634.

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

Glenner H, Hebsgaard MB. 2006. Phylogeny and evolution

of life history strategies of the parasitic barnacles (Crustacea, Cirripedia, Rhizocephala). Mol Phylogenet Evol 4:528–538.

Grube M, Hawksworth DL. 2007. Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. Mycol Res 111: 1116–1132.

Gueidan C, Roux C, Lutzoni F. 2007. Using a multigene analysis to assess generic delineation and character evolution in the Verrucariaceae (Verrucariales, Ascomycota). Mycol Res 111:1145–1168.

———, Ruibal Villaseñor C, Hoog GS, Gorbushina AA, Untereiner WA, Lutzoni F. 2008. A rock-inhabiting ancestor from mutualistic and pathogen-rich fungal lineages. Stud Mycol 61:111–119.

Hafellner J. 1984. Studien in Richtung einer naturlicheren Gliederung der Sammelfamilien Lecanoraceae und Lecideaceae. Nov Hedwig 79:241–371.

Henssen A. 1965. A review of the genera of the Collemataceae with simple spores (excluding *Physma*). Lichenologist 3:29–41.

———. 1979. New species of *Homothecium* and *Ramalodium* from S America. Bot Notiser 132:257–282.

———. 1981. The lecanoralean centrum. In: Reynolds DR, ed. Ascomycetes systematics. New York: Springer-Verlag. p 139–234.

———. 1999. New species of *Ramalodium* and *Staurolemma* from Australasia (Collemataceae, lichenized ascomycetes). Nova Hedwigia 68:117–130.

Hofstetter V, Miadlikowska J, Kauff F, Lutzoni F. 2007. Phylogenetic comparison of protein-coding versus ribosomal RNA-coding sequence data: a case study of the Lecanoromycetes (Ascomycota). Mol Phylogenet Evol 44:412–426.

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755.

Jørgensen PM, Henssen A. 1999. Further species of the lichen genus *Staurolemma* (Collemataceae, lichenized ascomycetes). Bryologist 102:20–25.

Kalendar R. 2005. FastPCR, PCR primer design, DNA and protein tools, repeats and own database searches program (www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm).

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Ainsworth and Bisby's Dictionary of the Fungi. 10th ed. Wallingford, UK: CABI International. 771 p.

Larsen N, Olsen GJ, Maidak BL, McCaughey MJ, Overbeek R, Macke TJ, Marsh TL, Woese CR. 1993. The ribosomal database project. Nucleic Acids Res 21: 3021–3023.

Larson DW. 1979. Lichen water relations under drying conditions. New Phytol 82:713–731.

Lumbsch HT, Huhndorf SM. 2007. Outline of Ascomycota 2007. Myconet 13:1–58.

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 49:628–651.

Maddison W, Maddison D. 2001. MacClade: analysis of phylogeny and character evolution, version 4.01. Sunderland, Massachusetts: Sinauer Associates.

———, ———. 2007. Mesquite v. 2.01 (http://mesquiteproject.org/mesquite/mesquite.html).

Mason-Gamer RJ, Kellogg EA. 1996. Testing for phylogenetic conflict among molecular datasets in the tribe Triticeae (Gramineae). Syst Biol 45:524–545.

Miadlikowska J, Kauff F, Hofstetter V, Fraker E, Grube M, Hafellner J, Reeb V, Hodkinson BP, Kukwa M, Lücking R, Hestmark G, Otálora MAG, Rauhut A, Büdel B, Scheidegger C, Timdal I, Stenroos S, Brodo I, Perlmutter G, Ertz D, Diederich P, Lendemer JC, Schoch C, Tripp E, Yahr R, May P, Gueidan C, Arnold AE, Robertson C, Lutzoni F. 2006. New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA and two protein-coding genes. Mycologia 98:1089–1102.

———, Lutzoni F. 2000. Phylogenetic revision of the genus *Peltigera* (lichen-forming ascomycetes) based on morphological, chemical and large subunit nuclear ribosomal DNA data. Int J Plant Sci 161:925–958.

———, ———. 2004. Phylogenetic classification of Peltigeralean fungi (Peltigerales, Ascomycota) based on ribosomal RNA small and large subunits. Am J Bot 91: 449–64.

Miller AN, Huhndorf M. 2005. Multi-gene phylogenies indicate ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology in the Sordariales (Ascomycota, Fungi). Mol Phylogenet Evol 35:60–75.

Otálora MAG, Martínez I, Molina MC, Aragón G, Lutzoni F. 2008. Phylogenetic relationships and taxonomy of the *Leptogium lichenoides* group (Collemataceae) in Europe. Taxon 57:907–921.

Pagel M, Meade A, Barker D. 2004. Bayesian estimation of ancestral character states on phylogenies. Syst Biol 53: 673–684.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 49:817–818.

Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycol Res 98:625–634.

Rodríguez F, Oliver JL, Marín A, Medina JR. 1990. The general stochastic model of nucleotide substitution. J. Theor Biol 142:485–501.

Ronquist F, Huelsenbeck JP. 2003. MrBayes v3.1.2 (Bayesian phylogenetic inference under mixed models). Bioinformatics 19:1572–1574.

Rundel PW. 1982. The role of morphology in the water relations of desert lichens. J Hattori Bot Lab 53:315–320.

Schmitt I, Lumbsch HT. 2004. Molecular phylogeny of the *Pertusariaceae* supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. Mol Phylogenet Evol 33:43–55.

———, Mueller G, Lumbsch HT. 2005. Ascoma morphology is homoplaseous and phylogenetically misleading

in some pyrenocarpous lichens. Mycologia 97:362–374.

Sierk HA. 1964. The genus *Leptogium* in North America and north of Mexico. Bryologist 67:245–317.

Souza-Egipsy V, Valladares F, Ascaso C. 2000. Water distribution in foliose lichen species: interactions between method of hydration, lichen substances and thallus anatomy. Ann Bot 86:595–601.

Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Vilgalys R, Hester M. 1990. Rapid identification and mapping of enzymatically amplified ribosomal DNA from several *Crytococcus* species. J Bacteriol 172:4238–4246.

Wiens JJ. 1998. Combining datasets with different phylogenetic histories. Syst Biol 47:568–581.

Wiklund E, Wedin M. 2003. The phylogenetic relationships of cyanobacterial lichens in the Lecanorales suborder Peltigerineae. Cladistics 19:419–431.

Zahlbruckner A. 1921–1930. Catalogus Lichenum Universalis. 10 Vols. Leipzig: Borntraeger.

Zoller S, Scheidegger C, Sperisen C. 1999. PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming ascomycetes. Lichenologist 31:511–516.