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Cardinal characters on a slippery slope - a re-evaluation of phylogeny, character evolution, and evolutionary rates in the jelly lichens (Collemataceae s. str)

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ABSTRACT

Recent molecular systematic studies have indicated that the traits currently used for generic delimitation in the jelly lichens (Collemataceae s. str.), may not characterize monophyletic groups. Here we reconstruct the phylogeny of Collemataceae using Bayesian and maximum likelihood analyses based on mitochondrial (mtSSU rDNA) and nuclear (nuLSU rDNA, Beta-tubulin and MCM7) markers of 70 Collemataceae species. We studied the evolution of four morphological and ecological characters traditionally used to delimit genera and infra-generic groups. Finally, we tested if differences in branch-lengths between clades are due to differences in rates of molecular evolution. Eleven strongly supported groups were recovered in the resulting well-resolved and well-supported phylogeny. The presence/absence of a eucortex, which is currently used as the cardinal character to define genera in the group, does not characterize monophyletic groups corresponding to the genera as currently circumscribed. Ancestral state reconstruction indicates that the most recent common ancestor of the jelly lichens most likely was saxicolous/terricolous, lacked a tomentum, and had transversally septate ascospores. Although the cortex state could not be reconstructed for the ancestor of the family, our observations indicate that a lack of cortex may have an evolutionary advantage in saxicolous/terricolous species in semi-arid environments, as non-corticate species tends to be larger and occur in higher frequency and abundance in such regions, compared to corticate species. A significant evidence for faster evolutionary rates was found in a lineage mainly including taxa that occur in the wet tropics and humid temperate regions, compared to other lineages. We suggest that this can explain the greater diversity of Collemataceae in tropical and humid areas.

Key words: Accelerated evolutionary rates, ancestral state reconstruction, Collemataceae, cortex, habitat, lichen-forming fungi.

1. Introduction

Lichens are fungi utilizing photosynthetic algae or cyanobacteria for the exploitation of carbohydrates. The symbiotic association allows both the fungal and algal partners to colonize a range of habitats which otherwise would be inhabitable to them. Molecular phylogenetic studies have recently led to significant advances in the understanding of relationships between fungal groups including lichen fungi (Lutzoni et al., 2004; Wedin et al. 2004; 2005; Miadlikowska et al., 2006; Hibbett et al., 2007; Ekman et al., 2008). These and other studies have provided strong evidence that many commonly used morphological and anatomical characters traditionally utilized to classify lichenized fungal taxa often are poor predictors of monophyletic groups. By contrast, traits traditionally considered less important in taxonomy are often better indicators of phylogenetic relationships in lichens (Schmitt et al., 2005; Blanco et al., 2006; Gueidan et al., 2007; Schmitt et al., 2009a; Rivas-Plata et al., 2011).

The Peltigerales is a group of fungi forming symbioses with cyanobacteria, and which earlier often were viewed as “ancient” and featuring many presumably “old” traits (Hawksworth, 1982; Galloway, 1994). These lichens have potentially a substantial impact on the ecosystems they inhabit by fixating atmospheric nitrogen (Cornelissen et al., 2007; Nash, 2008) and many species are rare and threatened by forestry management practices and air pollution. The Collemataceae (the “jelly lichens”) is a large group of distinctly gelatinous lichens within the Peltigerales. The gelatinous habit is due to a sheet forming around the colonies of the filamentous cyanobacterial symbiont (*Nostoc*) which swell and becomes extremely gelatinous when wet. In a traditional circumscription the members of the Collemataceae were also characterized by a similar ascoma development, a disc-shaped proper wall (exciple), and asci with an internal amyloid tube structure (Henssen, 1965; Henssen, 1981). Henssen (1965) included two genera with septate ascospores (*Collema* F.H.

Wigg. and *Leptogium* (Ach.) Gray), and six genera with single ascospores (*Homothecium* A. Massal., *Leciophysma* Th. Fr., *Leightoniella* Henssen, *Physma* A. Massal., *Ramalodium* Nyl. and *Staurolemma* Körb.) in this family. Recent phylogenetic studies, however, showed that the emphasis on the gelatinous thallus structure had resulted in a misguided classification and that all the genera with single-celled spores belong to another distinct clade of cyanobacterial Peltigerales, the Pannariaceae (Wedin et al., 2009; Otálora et al., 2010). Finally, a species that morphologically otherwise is similar to *Leptogium* was described as an *Arctomia* (Magain and Sérusiaux, 2012), and the *Collema* Fasciculare-group was recently also shown to belong to *Arctomia* s. lat. in the Ostropomycetideae (Otálora and Wedin, 2013). The remaining two genera with septate ascospores (*Collema* and *Leptogium*) together form a distinct monophyletic group that should correctly be classified as the Collemataceae, and which is characterized by a non-stratified (“homoiomorous”) thallus, a closed disc-shaped fruitbody wall (exciple) and a distinct ascus type similar to the *Micarea*-type, shared with Placynthiaceae, the closest relative (Spribille and Muggia, 2012).

Within the re-circumscribed Collemataceae, the generic classification is based on one morphological trait only. Species classified in *Leptogium* has thus a distinct cortex including at least one well-defined layer of cortical cells, while species classified in *Collema* lacks a cortex completely or develop an indistinct pseudo-cortex. Already Gunnar Degelius, the great monographer of *Collema*, had doubts about the monophyly of this genus and suggested that different species of *Leptogium* may have evolved from different species of *Collema* (Degelius, 1954). This was also supported by several early studies of Peltigerales phylogeny, where the included *Collema* and *Leptogium* representatives did not form monophyletic groups (Wiklund and Wedin, 2003; Miadlikowska and Lutzoni, 2004; Wedin and Wiklund, 2004; Miadlikowska et al., 2006). Two recent studies focussing more specifically on Collemataceae in the old, wide sense confirmed this reciprocal non-monophyly (Wedin et al., 2009; Otálora

et al., 2010), which was also supported by later studies on Peltigerales (Muggia et al., 2011; Spribille and Muggia, 2012).

Within *Collema* and *Leptogium* there is a high morphological and anatomical variation, and this is reflected by the infrageneric classifications currently in use (Table 1). Degelius (1974) subdivided *Collema* into 22 informal groups based on ecological and morphological characters as habitat, habitus, thallus size, texture and anatomy, and ascospore characteristics. *Leptogium* was subdivided by Zahlbruckner (1924) into 7 sections based on presence/absence of hairs on the thallus surfaces (“tomentum”), spore shape, thallus anatomy and cortex characteristics, but their taxonomic status is uncertain and only five of them have been recognized by later authors (Sierk, 1964; Verdon, 1992). More recently, *Leptogium* has also been subdivided in informal species groups or species complexes (Jørgensen, 1973, 1975, 1994, 1997; Jørgensen and James, 1983; Otálora et al., 2008), particularly within the largest section of the genus (section *Leptogium*). The preliminary phylogenetic investigations so far have not supported that these groupings reflect the evolutionary relationships between species (Wedin et al., 2009; Otálora et al., 2010). This indicates that most morphological characters traditionally used for generic and sub-generic classification are poor predictors of phylogenetic relationships. Otálora et al. (2010), using two molecular markers, suggested that Collemataceae includes at least five distinct clades, some containing representatives of both *Collema* and *Leptogium*. This study, however, included only a comparatively limited number of species, which did not cover the full diversity of the family, and could not identify any morphological characters reflecting the phylogenetic relationships. Nevertheless, these early Collemataceae phylogenies showed that the larger species of *Collema* and *Leptogium* group together in a single monophyletic clade. That clade of larger taxa also had comparatively longer branch-lengths than other clades in Collemataceae, and is the only group in the family that mainly includes taxa with a distribution in the wet tropics and humid temperate regions.

The rest of the tree mainly contains species with a predominantly temperate or semi-arid distribution.

Recently, the study of character evolution has dramatically increased our knowledge of how morphological and ecological traits evolve in lichens, and how these reflect phylogenetic relationships (Blanco et al., 2006; Gueidan et al., 2007; Ekman et al., 2008; Schmitt et al., 2009a; Muggia et al., 2011). In the Collemataceae, many of the easily observable and ecologically significant traits traditionally used for taxon circumscription are clearly not characterizing monophyletic groups. It is thus crucial to evaluate how the evolution of the group has resulted in the current distribution of such morphological characters and ecological preferences. Here, we will study the evolution of several such traits: the cortex, the tomentum, and the spore septation, and perform ancestral character state reconstructions. In addition, we will evaluate how well the distinct ecological preferences in the group reflect phylogenetic relationships. Finally, as earlier published Collemataceae phylogenies indicated that a clade mainly containing wet-tropical and moist-temperate taxa has comparatively longer branches than other lineages, and other studies have indicated that lichens with a similar distribution have higher rates of molecular evolution compared to groups with a predominantly temperate or semi-arid distribution (Lumbsch et al., 2008), we also wanted to test whether this clade exhibit faster rates of molecular evolution. If so, that would support the hypothesis that ecological shifts in climate zones and moisture regimes within the phylogeny of lichen fungal groups result in evolutionary rate discrepancies within clades, as was suggested in the parmelioid lichens (Lumbsch et al., 2008). We do this by conducting a detailed phylogenetic study covering the morphological and ecological diversity of *Collema* and *Leptogium*, increasing both the number of species and molecular markers considerably compared with our previous phylogenetic studies (Wedin et al., 2009; Otálora et al., 2010), to cover almost all earlier suggested infrageneric groupings within *Collema* and *Leptogium*.

2. Materials and methods

2.1. Taxon sampling

Our sampling aims at representing the full morphological, anatomical and ecological diversity within the family, and covers most of the previously recognized subgeneric groupings listed in Table 1. Some small, mainly monotypic groups of *Collema* were not represented in the sampling due to lack of recent material, and the *Collema* Fasciculare-group was excluded as it belongs to *Arctomia* s. lat. (Otálora and Wedin, 2013). Sequence data of the ribosomal genes mtSSU rDNA, nuLSU rDNA and the two protein-coding genes MCM7 and Beta-tubulin were obtained from a total of 101 Collemataceae samples, corresponding to 70 species. The geographic origin of the material, voucher specimens and GenBank accession numbers are presented in Table 2. *Parmeliella triptophylla* (Pannariaceae) was defined as outgroup to root the phylogeny. Representatives of the Placynthiaceae (the sister group of Collemataceae s. str.) and the Pannariaceae (a closely related family, where other taxa of Collemataceae s. lat. belong, Wedin et al., 2009) were in addition included, to evaluate the monophyly of the ingroup.

2.2. DNA sequencing

Total genomic DNA was extracted using DNeasy (Qiagen) according to the manufacturer's instructions. Total DNA was used to PCR amplification of fungal nuclear and mitochondrial loci. The nrLSU rDNA was amplified using the primers LR7 (Vilgalys and Hester, 1990), nrLSU 0170-R (Otálora et al., 2010) and the newly designed Collemataceae primers LR500ColIF (ACCAAGGAGTCTAACGTCCCCG) and LR1200ColIR (CATTCGGCCGGTGAGCTGTTACG); mtSSU rDNA using the primers mtSSU1 and mtSSU3R (Zoller et al., 1999). The protein coding Beta-tubulin gene was amplified using the

primers Bt3-LM, B10-LM (Myllys et al., 2001) and the newly designed Collemataceae primers BetaCollF (AGGCATCTGGAAACAAATATGT) and BetaCollR (GCGCCACGGCTTGTCAACG); and the locus MCM7 using the primers MCM7-709 and MCM7 1348 (Schmitt et al., 2009b). PCR amplifications were performed using Illustra™ Hot Start PCR beads, according to the manufacturer's instructions. PCR-reactions were performed using the following cycling parameters: initial denaturation 95°C for 5 min, and 35 (mtSSU) or 40 (nuLSU, MCM7) cycles at 95°C for 1min, 56°C for 50 sec (mtSSU) or 54°C for 1 min (nuLSU and MCM7), 72°C for 1 min; followed by a final extension at 72°C for 8 min. The PCR conditions for the Beta-tubulin began with an initial denaturation of 95°C for 5 min, followed by 4 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 8 min. PCR products were subsequently purified using the enzymatic method Exo-sap-IT (USB Corporation, Cleveland, OH). Unidirectional dye-terminated sequencing for the forward and reverse reactions using the same PCR primers were performed on an ABI 377 automated sequencer using Big Dye Terminator technology (Applied Biosystems, Warrington).

2.3 Phylogenetic methods

Alignments were constructed separately for each locus with the online version of MAFFT 6 using the auto option (Kato and Toh, 2008). The robustness of the nuLSU and mtSSU alignments was examined using GUIDANCE web service (Penn et al., 2010). Alignment regions with reliability higher than the default cut-off of 0.90 were used for further analyses and the rest were excluded as ambiguous. The alignments for the protein coding genes were adjusted in MacClade 4.01 (Maddison and Maddison, 2001) utilizing the amino acid translations.

Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian inference (MCMC). The combinability of the single locus data sets was assessed by comparison of the individual bootstrap values (Mason-Gamer and Kellogg, 1996; Wiens, 1998). A conflict was considered significant when two data partitions supported conflicting monophyletic group with ML bootstrap values $\geq 70\%$ in both trees. Because no significant conflicts were detected, it was assumed that the two data sets were congruent and could be combined. Maximum Likelihood was performed on the 4 locus dataset using RAxML-HPC2 (Stamatakis, 2006; Stamatakis et al., 2008) implementing a GTRCAT model for the bootstrapping phase, and GTRGAMMA for the final tree inference. The dataset was analysed using 8 partitions (three codon positions for each MCM7 and Beta-tubulin) on the Cipres Web Portal (Miller et al., 2010) and a bootstrap analysis of 1000 pseudoreplicates were performed.

The evolutionary models for Bayesian analyses were selected using the Bayesian Information Criteria (BIC) as implemented in JModeltest 3.06 (Posada, 2008). The GTR + I + G model was used for all partitions, except for mtSSU rDNA where the SYM + G was selected. The Bayesian inference was performed using MrBayes 3.2.0 (Ronquist et al., 2011). Two runs of 5 million generations starting from an initial random tree and employing four simultaneous chains were executed. A tree was saved every 100th generation. To ensure that stationarity was reached and the runs converged at the same log-likelihood levels, we plotted the log-likelihood scores of sample points against generation time using Tracer 1.5 (Rambaut, 2007). The first 12500 trees from each run were discarded as “burn-in”. For the remaining trees in each analysis, a majority rule consensus tree was assembled using the sumt option of MrBayes. The .con file created by MrBayes was visualized in FigTree V.1.2.2 (Rambaut, 2009).

2.4. Character evolution

The four characters studied were coded through own macro- and microscopic observations of specimens, and complemented by reliable literature statements from Degelius (1954, 1974), Sierk (1964), Jørgensen (1975, 1994, 1997, 2007), Jørgensen and Nash (2004), Aragón et al. (2005) and Galloway (2007). The cortex character was coded as 0= absent (Fig. 1a), 1 = eucortex (a very regular structure made up by a single layer of isodiametric cells, Fig. 1b) and 2= pseudocortex (an irregularly developed structure of small and flattened cells, Fig. 1c). The outgroups have different thallus cortex which we do not consider similar to any of the cortex character states in Collemataceae, and was thus coded as "inapplicable" in all outgroups. The tomentum character was coded as having three states: 0= absent, 1= hairs composed of cylindrical cells (Fig. 1d), and 2= hairs composed of spherical cells (Fig. 1e). Ascospore septation was coded as having 0= transversal septa (Fig. 1f-h), 1= longitudinal and transverse septa (Fig. 1i), 2 = no septa (outgroup). The only ecological character included was habitat preference: 0= predominantly epiphytic, 1= saxicolous and/or terricolous. It is difficult to assign many species to one type of habitat as they can be epiphytic, saxicolous and terricolous at the same time, and the distinction between saxicolous and terricolous is not easy. However, a large number of species have never been reported as epiphytic, and are thus here considered obligatory saxicolous/terricolous.

We inferred ancestral states and traced the evolution by employing two different methodologies using the last 10000 trees resulting from the combined Bayesian analysis. Parsimony ancestral state reconstruction was performed using Mesquite 2.01 (Maddison and Maddison, 2007). Ancestral reconstruction using a Bayesian approach was carried out using SIMMAP v.1. (Bollback, 2006). SIMMAP offers two priors, the overall rate and the two state bias parameter priors (Bollback, 2006). The overall substitution rate of each morphological character was modelled with a gamma distribution with parameters α and β . Additionally, for two-state morphological characters, SIMMAP requires a bias parameter, which follows a

symmetrical beta distribution and is described by the single parameter α (Bollback, 2006). Prior parameters were calculated using a two-step approach (Bollback, 2009). First, we performed an MCMC analysis in SIMMAP v. 1.5 (Bollback, 2006) to sample overall rate values (gamma and beta priors). Second, to find the parameters that best fit the gamma and beta distributions, the results from the first step were analysed with the R Statistical Package and the script provided with SIMMAP v. 1.5. Thus we obtained the follow priors to be used in the ancestral state reconstruction analyses. Overall rate priors: cortex ($\alpha = 1.5$ $\beta = 0.6$), tomentum ($\alpha = 2.2$ $\beta = 0.4$), ascospores septation ($\alpha = 3.1$ $\beta = 0.3$), habitat preference ($\alpha = 1.6$ $\beta = 0.5$) all with 60 categories; bias parameter: ascospores septation ($\alpha = 1.4$), habitat preference ($\alpha = 1.0$) with 31 categories.

2.5. Molecular evolution rate differences test.

To test if the lineage mainly including species occurring in the tropical and moist temperate zones has a different rate of mutation accumulation compared to other lineages, we used the program BaseML of the PAML package (Yang, 2007). We compared the likelihoods among a simple model where the entire Collemataceae phylogeny experienced the same rate of evolution (null model) to a more complex alternative model where this lineage was allowed to evolve at a different rate than the rest of the lineages in the tree. The comparisons were made in the individual and combined datasets. The resulting likelihoods from each model were compared using the likelihood ratio test.

3. Results

3.1 Phylogenetic analyses

The four-locus data set contained 3028 unambiguously aligned sites of which 1109 were variable. Of the variable sites 288 belonged to the mtSSU rDNA partition, 330 to the nuLSU rDNA, 261 to MCM7 and 230 to Beta-tubulin. The percentages of missing data were

1.1, 18.5, 1.8, and 9.5 for mtSSU, nuLSU, MCM7 and Beta-tubulin respectively. Maximum Likelihood analysis resulted in a single most likely tree with a ln-likelihood of -23658.318. The majority rule consensus tree from the Bayesian analysis was based on 75000 credible trees from two runs. The harmonic mean ln-likelihood was -23779.70. The tree topologies obtained by the maximum likelihood and a Bayesian approaches did not show any supported conflict and therefore only the 50% majority rule consensus tree from Bayesian analysis is shown, with both bootstrap support and posterior probabilities (Fig. 2).

As in earlier investigations, both *Collema* and *Leptogium* are non-monophyletic in their current circumscriptions. Collemataceae is composed of eleven clades (A-K), which are strongly supported in both the ML and Bayesian analyses (Fig. 2). One of these, clade I, includes species of both *Collema* and *Leptogium*. Eight clades includes only species currently classified in *Collema* (clades A, D, E, F, G, H, J) and three clades includes only current species of *Leptogium* (clades B, C, K). The relationships between all these clades are very well supported in both analyses, with the exception of the node uniting clades A and B.

3.2. The reconstruction of ancestral character states

Ancestral states of the cortex (Fig. 1a-c), tomentum (Fig 1d-e), ascospore septation Fig. 1f-h) and habitat preference characters were reconstructed for the Collemataceae node (Fig. 3, node 39), the 11 clades defined above, and for other internal nodes depending on what was seen as interesting and informative for the character (Fig. 3). Posterior probabilities (PP) at each state at each node from the two methodologies (Parsimony and Bayesian inference) are shown in Table 3. Posterior probabilities > 0.95 were considered significant for a state of a clade and the reconstruction was interpreted as uncertainty when it was not supported by the two methodologies.

Bayesian reconstruction suggests that the most recent ancestor of Collemataceae (Node 39) was most likely saxicolous/terricolous, lacking tomentum, and having transversally septate ascospores. The cortex state could not be significantly reconstructed for the ancestor of the family, but an early transition must have occurred, as the ancestor of clade K is reconstructed as eucorticate (node 38) and the ancestor of its sister lineage (node 37) as non-corticate (Fig. 3, Table 3). Parsimony suggests five independent gains of eucortex (Fig. 1b); three of which occurred within clade I (nodes 23, 28, 29), and the others in clades B (node 12) and C (node 10). Four of the five transitions most likely occurred from a non-corticate ancestor, while one (node 23) evolved from an ancestor (node 25) with pseudocortex (Fig. 3, Table 3). The pseudocortex state (Fig. 1c) has evolved once from the non-corticate state, in a group within clade I (node 25). The Bayesian reconstruction show a different scenario with four significant gains of eucortex, one early in the ancestor of the lineage formed by clades A, B and C (node 13), and three more recent within clade I (nodes 23, 28, 29).

Bayesian and Parsimony reconstructions support four recent gains of a tomentum of hairs with cylindrical cells (Fig. 1d) from a non-tomentose ancestor, in the ancestor of clade C (node 10), and independently in three groups within clade B (nodes 7, 8, 9). The origin of a tomentum of spherical cells (Fig. 1e) was significantly reconstructed once only, at node 6 in clade B.

The Bayesian method reconstructed transversal septate ascospores (Fig. 1f-h) as the ancestral spore character state for the family (node 39). Six independent transitions to muriform ascospores (Fig. 1i) were reconstructed by Bayesian inference and Parsimony in the ancestors of clade B, C, F, K, (node 12, 10, 18 and 38, respectively), in the lineage formed by clades I and J (node 33) and within clade A (node 3). Only one transition from muriform to transversal septate ascospores has occurred, within clade B (*L. biloculare*).

Two transitions from the saxicolous/terricolous to the epiphytic lifestyle were reconstructed, both by the Bayesian and parsimony methods; in the common ancestor of clades A, B, C, and D (node 14), within clade F (node 19). Bayesian inference reconstructed significantly one more transitions in the ancestor of clade I (node 32), followed by two transitions back to saxicolous/terricolous state (node 25, 31). Parsimony did not support the transition in node 32 but suggested that the transitions to epiphytic lifestyle occurred three times within clade I (nodes 27, 28, 29).

3.3 Difference in sequence evolution rate

The clade ABC (Fig. 2) has comparatively longer branch-length than the remaining eight clades (D-K). Clade ABC contains taxa that occur in the wet tropics and humid temperate regions, compared to the remaining eight clades (D-K) which mainly contain taxa that occur predominantly in temperate, mainly sub-arid regions. The alternative model, where the clade ABC evolved at a different rate compared to the rest of the lineages in the tree, was found to be significantly better than the null model, where the entire Collemataceae phylogeny evolved at the same rate (Table 4). This was the case in single-marker analyses of the ribosomal markers and the analysis of the combined dataset, but not in single-marker analyses of the protein-coding genes.

4. Discussion

4.1. Phylogenetic relationships in Collemataceae

The four-locus data set produced a highly resolved and well supported topology, resolving numerous new relationships compared with previous Collemataceae phylogenies that included fewer markers and a more restricted taxon sampling (Wedin et al., 2009; Otálora

et al., 2010). Most of the backbone topology is very well supported both by the Bayesian and the ML analyses.

The first clade, clade A (Fig. 2), contains the epiphytic, larger species of *Collema*, and includes the generic type (*Collema nigrescens*). This clade comprises the species of four morphologically similar informal infra-generic *Collema* units (the Nigrescens, Japonicum, Leptaleum, and the Coilocarpum groups) defined by Degelius (1974). With one exception (*C. subconveniens*) all the species in this clade have transversely septate ascospores. The relationships observed in clade A are consistent with the findings by Otálora et al. (2010), who only included member of the Nigrescens and Japonicum groups. Its sister clade, clade B, comprises large and mainly epiphytic species of *Leptogium*, almost all of them having muriform ascospores (the one exception is *L. biloculare*). The backbone topology in clade B is however not well supported or have support only from the Bayesian analysis (Fig.2). Clade B includes species currently classified in the sections *Leptogium*, *Mallotium*, and *Leptogiopsis*, all of which are non-monophyletic in our analyses. A good example of an earlier recognised morphological group which is scattered along this clade is a species complex suggested by Jørgensen and James (1983), here represented by *Leptogium azureum*, *L. britannicum*, *L. cyanescens* and *L. rivulare*. They are nested in three different parts of the clade B, together with species belonging to other species complexes (*i.e.* *L. phyllocarpum*; Fig.2). Another example of a morphologically defined and widely accepted group that is here shown to be non-monophyletic, is *Leptogium* section *Mallotium*. This section is characterized by the presence of a tomentum. The species where the tomentum is composed by hairs with spherical cells (Fig. 1e) form a distinct monophyletic group (*Leptogium digitatum*, *L. hibernicum*, *L. laceroides*, *L. velutinum*) within clade B, while species with a tomentum composed by hairs with cylindrical cells (Fig. 1d) form three groups, two of which are found within clade B. One of these (*L. resupinans*, *L. juressianum*) is the sister to the group with

spherical-celled hairs, and four other species form a poorly supported group (*L. pseudofurfuraceum*, *L. papillosum*, *L. furfuraceum* and *L. malmei*). *Leptogium resupinans* and *L. juressianum* both have tomentum only on the upper surface while the rest of the tomentose species mainly produce it on the lower surface. The remaining *Mallotium* species with cylindrical hairs, including *Leptogium saturninum*, the type of *Mallotium*, comprise Clade C. Finally *L. brebissonii*, which is represented here by one sample from Europe and one from South America, does not form a monophyletic unit. This may well be an earlier undetected taxonomic problem.

Although clades A, B, and C together form a well-supported lineage containing large epiphytic *Collema* and *Leptogium* species, there are no obvious morphological connections between clade A and the other two. Species in clade A are characterized by the absence of a thallus cortex, and by having spores with only transversal septa. Species in clades B and C have a thallus with a well-developed eucortex in both the lower and upper surfaces, and the spores are mainly muriform. The lineage (ABC) has the largest number of species, and is the only lineage that comprises species that are mainly distributed in the wet tropics. This lineage, also characterized by longer branches, was already detected in previous phylogenies (Wedin et al., 2009; Otálora et al., 2010).

The sister to this lineage, clade D, contains the small corticolous *Collema italicum*, which is characterized by having foliose thalli with very small asci and ascospores. This combination of morphological characteristics only occurs in the small *Collema italicum*-group (Degelius, 1974), which includes two species distributed in Southern Europe and Northern Africa.

Clade E is formed by species of the *Collema* Tenax-group, which is characterized by swollen and plicate thallus lobes, and apothecia that usually have a distinct anatomy of the exipulum proprium (euthyplechtenchymatous). Most are saxicolous or terricolous, with some

single exceptions (*C. conglomeratum* and *C. ligerinum*). *Collema polycarpon*, represented here by one European and one South American specimen, is paraphyletic. Contrary to the species forming clade A-D, which are predominantly epiphytic and grow in shady situations, the Tenax-group mainly contains pioneer species growing on bare soil or rocks, preferably in open and exposed habitats. Although the species of the Tenax-group are cosmopolitan, they are much more prevalent in temperate region than in the tropics, and occur in comparatively dry areas. Clade E is the strongly supported sister to the lineage formed by clades A, B, C and D. Otálora et al. (2010) already suggested this sister-group relationship, but in their study it lacked support.

The clade F comprises species of the *Collema* Occultatum-group, which is characterized by having very small thalli and cubic muriform ascospores. Trevisan (1880) recognized this species group as the genus *Rostania* Trev. However, later authors continued to treat these species within *Collema* (Degelius, 1954). The Occultatum-group is mainly distributed in the temperate regions of the Northern hemisphere (Europe and North America) with some representatives in subtropical region of Asia. Clade G is formed by the *Collema* Multipartitum-group which is characterized by having deeply and repeatedly branched convex lobes, and transversely septate, linear to oblong ascospores. This species group is restricted to Europe, Northern Africa and North America. Although clade F and G together form a well-supported monophyletic lineage, there are no obvious morphological characteristics shared by these two groups (Fig. 3). This sister-group relationship is reported here for the first time.

The *Collema* Crispum-group (clade H) is characterized by having a very distinct thallus anatomy (homoiomorous but partially paraplectenchymatous). This group includes taxa with a world-wide distribution but predominantly occurring in temperate regions. The position of this group within *Collema* was questioned, because it is the most deviant species group regarding thallus anatomy in *Collema* (Trevisan, 1853; Degelius, 1954). Clade H is sister to a

lineage composed by clades I and J. As previous Collemataceae phylogenies did not include *C. crispum* (Wedin et al., 2009; Otálora et al., 2010), the phylogenetic position has so far been uncertain.

Clade I is formed by a number of relatively small-sized *Collema* and *Leptogium* species, including the generic type of *Leptogium* (*L. lichenoides*). This clade includes species of three different *Leptogium* sections (*Homodium*, *Collemodium* and *Leptogium*) and three *Collema* groups (*Fragrans*, *Callopismum* and *Leptogioides*). This clade is very heterogeneous regarding morphology, including species with different types of thallus anatomy. The saxicolous *C. parvum* and *C. callopismum* are nested in a well-supported sub-clade with species of the *Leptogium* sections *Leptogium* and *Collemodium*. The small, corticolous species *C. fragrans* is sister to them. Representatives of *Leptogium* section *Homodium*, characterized by having a compact medulla (paraplectenchymatous), form two distinct groups within clade I, both grouping with species of the section *Leptogium* (with lax medulla). The included samples of *Leptogium subtile* did not form a monophyletic unit, but as one sample lacks nuLSU and MCM sequences data, we interpret this as due to missing data. It is noteworthy that this is morphologically the most diverse clade; however, it exhibits shorter branch lengths than the other groups in the tree. The species in this clade are mainly restricted to temperate regions of the northern hemisphere, and are very rare in tropical regions.

Clade J contains the saxicolous medium-sized to large *Collema* species with muriform ascospores, all belonging to the *Collema Cristatum* group (Degelius, 1954). This group comprises some species with world-wide distribution, but all are more frequent in temperate regions. The lineage formed by clades I and J was already reported in previous phylogenies, but the sister relationship between these two clades lacked support (Otálora et al., 2010).

Leptogium diffractum (*Leptogium* section *Pseudoleptogium*), finally, is the sister (clade K) to the rest of the Collemataceae. *Leptogium diffractum* was already recognised as very

isolated by Müller Argoviensis (1885), who suggested the genus *Pseudoleptogium* for it, based on the distinct thallus habit (placoid-squamulose) and anatomical structure (paraplectenchymatous throughout). *Leptogium diffractum* was also sister of the rest of the Collemataceae in Wedin et al. (2009).

4.2. The reconstruction of ancestral character states

We mainly found differences between the results from the two approaches utilized in those cases where Mesquite (parsimony) could not significantly reconstruct a state for a given node. A well-supported incongruent result was, however, obtained in the reconstruction of the cortex character in node 13 (Table 3).

4.2.1. Cortex

Different functions have been attributed to the cortex in lichens (Grube and Hawksworth, 2007), and cortex characteristics have generally been regarded as key traits for classification in Collemataceae. The cortex state of the common ancestor of Collemataceae (node 39) is not recovered with certainty with any of the methods. The two most basal nodes above node 39 have well-supported cortex states, where node 38 (*L. diffractum*) is reconstructed as having a eucortex (Fig. 1b) and node 37 as having no cortex (Fig. 1a). Although the number of transitions from a non-corticate to a eucorticate state differ between the parsimony and Bayesian reconstructions, both methods agree in that transitions to a eucortex state occurred in lineages with an epiphytic ancestor (one exception is *L. diffractum*). Few *Leptogium* species are strictly saxicolous/terricolous, but all are grouped in subclades which have an epiphytic ancestor under the Bayesian reconstruction (Fig. 3, Table 3). The only transition to the pseudocortex state (Fig. 1c) occurred in node 25, and is associated with one of the few transitions from the epiphytic to the saxicolous/terricolous habitat state. The

strictly saxicolous/terricolous non-corticate species (i.e. clade E, G, H and J) tend to be larger and more frequent and abundant in semi-arid environments and exposed microhabitats (bare rocks and soil), than strictly saxicolous/terricolous species with a eucortex (e.g. *L. tenuissimum* and *L. schraderii*, in clade I). This could indicate that the lack of a cortex has an evolutionary advantage in the saxicolous/terricolous species. Although the absence of cortical layers increases the rate of water loss by evaporation (Jonsson et al., 2008), it also allows the thallus to take up water rapidly, favouring the increase of thallus size in an evolutionary perspective (Blum, 1973; Rundel, 1982; Lange et al., 1998; Souza-Egipsy et al., 2000). The relation water uptake-loss is crucial for the metabolic activity of the lichens, especially for the photosynthetic (photobiont) and respiratory activities (Qiu and Gao, 2001). Thus non-corticate species rapidly store larger amount of water, having more time available for metabolic activities before desiccation compared to eucorticate species. The gain of a eucortex, on the other hand, seems to be effective in foliose species that inhabit shady habitats (Clade B and C). In this type of habitat, there are more and more abundant eucorticate than non-corticate species, and eucorticate species inhabiting shady places also tend to be larger than species growing in exposed habitats. Although our observations suggest that absence of a thallus cortex may result in larger thallus size and abundance of such species in open and sub-arid habitats, detailed eco-physiological experiments are needed to investigate how cortex structure and thallus size vary among different ecological conditions.

4.2.2. *Tomentum*

Tomentum hairs evolved within the lineages containing large epiphytic and eucorticate species (Clades B and C). As hairs develop from a cortex, the evolution of the tomentum character states is obviously linked to the presence of a cortex. The tomentum composed by spherical cells (Fig. 1e) clearly represents a synapomorphy, evolving in node 6 only.

Although we include a large number of tomentose species in our study, a large number still remain to be studied. More detailed phylogenetic studies with a larger sampling are thus needed to fully understand the relationship among the tomentose and non-tomentose species in clade B.

4.2.3. *Ascospore septation*

The ascospore septation state reconstruction for the most internal clades is uncertain and the only significant posterior probability was obtained with Bayesian analysis, suggesting that the ancestor of the internal lineages (nodes 39, 37, 35, 15) had transversal septate ascospores (Fig. 1f-h). The evolution to muriform ascospores (Fig. 1i) took place in all lineages containing eucorticate species (*i.e.* *Leptogium* species) and in only two lineages containing non-corticate species (*Collema* species).

4.2.4. *Habitat*

The obligatory terricolous/saxicolous condition was the ancestral state of most of the internal nodes. The switch to the epiphytic lifestyle was very successful in the lineage formed by clades A-D, which mainly include foliose species. In clade I the epiphytic condition was also acquired (node 32), but a rapid transition back to terricolous/saxicolous condition apparently took place. Contrary to clade B and C where the epiphytic condition is almost obligatory, in Clade I the epiphytic condition is more facultative, and most of the species in reality grow over mosses on both trees and rocks.

4.3. *Difference in sequence evolution rate*

Significant differences in evolutionary rates between clade ABC and the remaining eight clades were detected when analysing the combined dataset (Table 4). The individual-

marker analyses showed that the differences are strongly significant in the nuLSU rDNA, weakly significant in the mtSSU rDNA, and not significant in the protein coding gene analyses. These results indicate that the ribosomal RNA genes have higher rates of heterogeneity among lineages than protein coding genes. This may not be unexpected as these genes include more insertions (DNA regions highly affected by mutations) compared with other genes, in fungi (Bhattacharya et al., 2000; DePriest, 2004; Simon et al., 2005; Hofstetter et al., 2007), and some of such insertions may have remained in our final dataset.

Heterogeneity in substitution rates among lineages is a common observation in molecular phylogenetic studies (Lutzoni and Pagel, 1997; Allen et al., 2006; Lumbsch et al., 2008; Smith and Donoghue, 2008). In the present study, we found significant rate heterogeneity between the wet-tropical/moist-temperate clade ABC and the rest of the lineages (Table 4), which together with the comparatively longer branches in clade ABC, suggests that this clade evolves faster than the other lineages. Rate heterogeneity between tropical and non-tropical lineages was earlier detected in the parmelioid lichens (Lumbsch et al., 2008). Several factors have been proposed to affect rate heterogeneity, such as organism size, metabolic rates, generation time and climatic conditions (Wright et al., 2003; Gillooly et al., 2005; Smith and Donoghue, 2008). However, to distinguish between these factors is difficult because they are often highly correlated. First, metabolic rate tend to be proportional to mutation rate, as a large proportion of the genetic damage is caused by sub-products of the metabolism (Gillooly et al., 2005). Second, metabolic rates vary depending on generation time, which in turn varies with organism size and environmental temperature (Allen et al., 2006). In the case of lichens, their metabolic and growth activities are restricted to periods when the thallus is hydrated from atmospheric water sources, such as rain, fog and high relative humidity (Rundel, 1982); therefore their metabolic rates are highly correlated with climatic factors. Although no information on generation time and metabolic rates is available

for the Collemataceae, environmental conditions in tropical and moist temperate areas presumably have a positive effect on the metabolic rates affecting mutation rates. This would explain the relatively higher substitution rate in clade ABC. Accelerated rates have been reported in tropical lineages of several organisms (Cardillo, 1999; Wright et al., 2003; Davies et al., 2004; Allen et al., 2006; Mittelbach et al., 2007; Lumbsch et al., 2008).

The increase of biodiversity along a latitudinal gradient, from the poles to the tropics, is one of the oldest recognized patterns in biogeography (Wallace, 1878; Fischer, 1960). This seems to be the case also in at least some groups of lichenized fungi (Sipman and Aptroot, 2001; Lücking et al., 2009; Lücking, 2012). In Collemataceae such a diversity gradient is also present (Sierk, 1964; Degelius, 1974; Verdon, 1992), where we know that clade ABC comprises the most species-rich groups in the family. We suggest that this increased diversity is caused by an increased speciation rate, which would be due to the accelerated evolutionary rate detected here, in combination with the increased rates of biotic and abiotic interactions that occurs towards the tropics (Schemske, 2009). The climatic environmental factors towards the equator are likely to be favourable to the gelatinous cyanobacterial lichens, which would benefit from constant high levels of temperature and moisture, and frequent spells of liquid water. Many recent studies have shown a positive correlation between rate of molecular substitution and speciation in a large range of taxa (Barracough and Savolainen, 2001; Webster et al., 2003; Pagel et al., 2006; Lancaster, 2010; Lanfear et al., 2010). However, only few studies have demonstrated a correlation between environmental factors (climate) and the relationship of molecular substitution rates and speciation rates (Davies et al., 2004; Mittelbach et al., 2007). Although this correlation seem to be present in Collemataceae, future detailed studies are needed to investigate the degree of how climate and other environmental variables drive substitution rates and thereby speciation in Collemataceae.

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Captions

Fig. 1. Light micrographs illustrating the character states studied. (A-C) Type of cortex. (A) Cortex absent in *Collema fragrans* (Aragón 793/96, MA). (B) Eucortex in *Leptogium digitatum* (Otálora 010190, S). (C) Pseudocortex in *L. plicatile* (Otálora 021012, S). (D, E) Tomentum type. (D) Hairs composed of cylindrical cells in *L. pedicellatum* (Thor 17186, UPS). (E) Hairs composed of spherical cells in *L. digitatum* (Otálora 0100190, S). (F-I) Ascospore septation. Ascospores with transversal septa in (F) *C. polycarpon* (Otálora 410512, S), (G) *C. laeve* (Wedin 8723, S), (H) *C. nigrescens* (Aragón 80/04, MA). (I) Ascospores with longitudinal and transverse septa in *L. lichenoides* (Aragón 1301/02, MA).

Fig. 2. The 50% majority rule consensus phylogram from the Bayesian analysis of the combined data matrix (nuLSU, mSSU, MCM7 and Beta-tubulin). PP values > 0.95 obtained in the Bayesian analysis and ML bootstrap values >70 are indicated above the branches. Supported values are ordered as PP/ML BS. The classification indicated at the right corresponds to Degelius (1974) *Collema* groups and the *Leptogium* sections of Zahlbruckner (1924). The monophyletic groups discussed in the text are indicated by letters (A-K).

Fig. 3. Character state distribution and nodes (1-39) used for ancestral state reconstruction, of four selected characters. The functional outgroups are coded as inapplicable in the cortex character as this structure is not homologous to any of the states present in Collemataceae. Branches in boldface indicate a support of PP > 0.95 and ML BS > 70%. Boxes correspond to monophyletic clades identified in Fig. 2.

Figure 1.

Figure 2.

Figure 3.

Table 1

Overview of the subgeneric classification currently in use in *Collema* and *Leptogium*.

Collema F.H. Wigg.		Leptogium (Ach.) Gray	
Degelius (1974) informal groups	Species included in this study	Zahlbruckner (1924) formal sections	Species included in this study
Tenax (9)	3	Sect. <i>Collemodium</i> (ca. 6)	2
Texanum (1)		Sect. <i>Pseudoleptogium</i> (1)	1
Italicum (2)	1	Sect. <i>Leptogiopsis</i> (ca. 4)	1
Redundans (1)		Sect. <i>Leptogium</i> (ca. 70)	22
Occultatum (5)	3	Sect. <i>Diplothallus</i> (1)	
Callopismum (1)	1	Sect. <i>Homodium</i> (ca. 15)	5
Leptogioides (4)	1	Sect. <i>Mallotium</i> (ca. 25)	14
Pustulatum (1)			
Crispum (3)	1		
Santessonii (1)			
Fragrans (3)	1		
Cristatum (7)	4		
Du-Rietzii (2)			
Leptaleum (3)	1		
Multipartitum (1)	1		
Japonicum (11)	3		
Coilocarpum (2)	1		
Hookeri (1)			
Nigrescens (10)	5		
Shiroumanum (1)			
Substipitatum (1)			
Fasciculare (4)			

Number in brackets correspond to number of species accepted in that sub-generic entity

Table 3

Ancestral states probabilities four diagnostic characters reconstructed using Bayes (Simmap) and parsimony. For reconstructed nodes see Fig. 3.

I Cortex

Node	Bayes (SIMMAP)			Parsimony (Mesquite)		
	non-cortex	eu cortex	pseudocortex	non-cortex	eu cortex	pseudocortex
2	1,000	0,000	0,000	1,000	0,000	0,000
10	0,000	1,000	0,000	0,000	1,000	0,000
11	1,000	0,000	0,000	1,000	0,000	0,000
12	0,000	1,000	0,000	0,000	1,000	0,000
13*	0,014	0,986	0,000	1,000	0,000	0,000
14	0,958	0,042	0,000	1,000	0,000	0,000
15	1,000	0,000	0,000	1,000	0,000	0,000
16	1,000	0,000	0,000	1,000	0,000	0,000
17	1,000	0,000	0,000	1,000	0,000	0,000
21	1,000	0,000	0,000	1,000	0,000	0,000
23	0,000	1,000	0,000	0,000	1,000	0,000
25	0,000	0,000	1,000	0,000	0,000	1,000
26	0,823	0,012	0,165	0,416	-	-
27	1,000	0,000	0,000	1,000	0,000	0,000
28	0,000	1,000	0,000	0,000	1,000	0,000
29	0,000	1,000	0,000	0,000	1,000	0,000
32	0,275	0,723	0,001	0,395	0,001	0,014
33	0,982	0,018	0,000	1,000	0,000	0,000
34	1,000	0,000	0,000	1,000	0,000	0,000
35	0,999	0,000	0,000	1,000	0,000	0,000
37	1,000	0,000	0,000	1,000	0,000	0,000
38	0,000	1,000	0,000	0,000	1,000	0,000
39	0,790	0,200	0,001	-	-	-

II Tomentum

Node	Bayes (SIMMAP)			Parsimony (Mesquite)		
	absent	cylindrical	spherical	absent	cylindrical	spherical
5	0,027	0,793	0,181	-	-	-
6	0,000	0,000	1,000	0,000	0,000	1,000
7	0,000	1,000	0,000	0,000	1,000	0,000
8	0,000	1,000	0,000	0,000	1,000	0,000
9	0,000	1,000	0,000	0,000	1,000	0,000
10	0,000	0,999	0,000	0,000	1,000	0,000
12	0,997	0,026	0,000	0,999	0,011	0,000
14	0,999	0,000	0,000	1,000	0,000	0,000
37	1,000	0,000	0,000	1,000	0,000	0,000
39	1,000	0,000	0,000	1,000	0,000	0,000

III Ascospore septation

node	Bayes (SIMMAP)		Parsimony (Mesquite)	
	transversal	muriform	transversal	muriform
2	0,999	0,001	1,000	0,000
3	0,000	1,000	0,000	1,000
10	0,013	0,999	0,000	1,000
11	1,000	0,000	1,000	0,000
12	0,049	0,951	0,000	1,000
13	0,788	0,212	-	-
14	0,980	0,020	0,780	0,220
15	0,982	0,018	0,755	0,240
16	0,980	0,010	0,760	0,240
18	0,042	0,958	0,000	1,000
20	1,000	0,000	1,000	0,000
21	1,000	0,000	1,000	0,000
32	0,001	0,999	0,000	1,000
33	0,018	0,982	0,000	1,000
34	0,010	0,990	0,000	1,000
35	0,953	0,047	0,754	0,250
37	0,975	0,024	0,754	0,250
38	0,000	1,000	0,000	1,000
39	0,961	0,039	0,754	0,250

Node	Bayes (SIMMAP)		Parsimony (Mesquite)	
	epiphytic	saxicolous /terricolous	epiphytic	saxicolous /terricolous
2	1,000	0,000	1,000	0,000
10	1,000	0,000	1,000	0,000
12	1,000	0,000	1,000	0,000
14	0,990	0,000	1,000	0,000
15	0,103	0,897	0,000	1,000
16	0,000	1,000	0,000	1,000
18	0,115	0,885	0,000	1,000
19	1,000	0,000	1,000	0,000
21	0,000	1,000	0,000	1,000
25	0,040	0,960	0,000	1,000
27	1,000	0,000	1,000	0,000
28	1,000	0,000	0,980	0,000
29	1,000	0,000	1,000	0,000
31	0,000	1,000	0,000	1,000
32	0,951	0,049	0,550	0,450
34	0,000	1,000	0,000	1,000
35	0,000	1,000	0,000	1,000
37	0,000	1,000	0,000	1,000
38	0,000	1,000	0,000	1,000
39	0,000	1,000	0,000	1,000

*node where reconstruction was incongruent (different result with different method).

Table 4

Likelihood comparison among two different models

Dataset	-Ln likelihood null model	-Ln likelihood alternative model	Likelihood radio test	DF	P value
mtSSU	6669.8926	6608.10233	123.5805	99	0.04779
nuLSU	7446.6778	7379.18288	134.9898	88	<0.0001
MCM7	8734.0364	8731.95180	4.169198	68	1.000
Beta-tubulin	9854.6328	9853.98290	1.299798	60	1.000
Combined	23978.3284	23912.3975	131.86173	105	0.03914

Null model: same rate of evolution in all lineages

Alternative model: Clade ABC (Fig. 2) has a different rate of evolution compared to the other lineages.