Phylogenetic significance of morphological characters in the tropical Hypotrachyna clade of Parmelioid lichens (Parmeliaceae, Ascomycota)

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Abstract

Lichen-forming ascomycetes exhibit often complex morphologies of the vegetative thallus that are usually not found in non-lichenized fungi. This includes the thallus organization and appendical structures associated with the main thallus, such as cilia and rhizines. Such morphological characters are widely employed in the taxonomy of Parmelioid lichens, especially at the generic level. Within Parmelioid lichens, several monophyletic groups can be distinguished, the Hypotrachyna clade being one of them, which includes mostly tropical taxa. In this first molecular study focused specifically on the Hypotrachyna clade, we used maximum parsimony and Bayesian analyses of a combined data set of nuclear ITS and mitochondrial SSU rDNA sequences to (1) test the monophyly of genera presently accepted within the clade and (2) evaluate the phylogenetic value of the morphological characters used to circumscribe genera in Parmelioid lichens. Out of the 89 mtSSU and 88 nuITS sequences included in the present study, 121 sequences were newly obtained. Our results show that the taxa within the clade fall into two major groups and that the genus Hypotrachyna is polyphyletic. Everniastrum and Parmelinopsis are nested within Hypotrachyna sensu stricto, the latter being also polyphyletic. Bulbothrix is paraphyletic with Parmelinella nested within and is basal to the second major Hypotrachyna clade. Monophilies of Bulbothrix and Hypotrachyna are significantly rejected. The phylogenetic analysis demonstrates that morphological characters currently used to circumscribe genera in Parmelioid lichens, such as cortical anatomy, lobe configuration, cilia, and rhizines have been overestimated and have only minor value in identifying monophyletic groups.

Keywords: Ascomycota; Combined analysis; Hypotrachyna; Lichens; Morphology; Parmeliaceae; Phylogeny

1. Introduction

Lichens are the symbiotic phenotype of lichen-forming fungi and green or brown algae or cyanobacteria. The symbiotic relationship between the fungal and photosynthetically active partner is often so well established that a special lichen thallus is formed. It is quite different in appearance from the morphology of single partners. This includes anatomical and chemical characters that are believed to be adaptive for these symbiotic organisms, such as pores in the cortical layers that facilitate gas exchange through the cortex (Beltman, 1978; Green et al., 1981, 1985; Hale, 1973, 1981; Yoshimura and Hurutani, 1987) or UV-absorbing compounds or pigments screening visible light and UV (Begora and Fahselt, 2001; Rikkinen, 1995; Solhaug and Gauslaa, 1996; Solhaug et al., 2003). Previously we have studied the evolution of these characters in the higher-level phylogeny of Parmelioid lichens (Blanco et al., 2006). However, a number of further morphological and chemical characters have been widely employed to circumscribe genera in lichenized fungi, especially in families with numerous foliose taxa, such as Parmeliaceae or Physciaceae (Elix, 1993; Elix and Hale, 1987; Hafellner et al., 1979; Hale, 1974ab, Nordin and Mattsson, 2001; Scheidegger et al., 2001). This includes mainly characters relating to thallus organization and appendical structures associated with the
main thallus, such as cilia and rhizines. The taxonomic significance of such vegetative characters has been disputed. While in the last few decades a number of genera in Parmelioid lichens have been described based on vegetative morphological and chemical characters (Culberson and Culberson, 1981; Elix, 1993; Elix and Hale, 1987; Elix et al., 1986; Hale, 1974b, 1984a,b, 1986; Krog, 1982; Kurokawa, 1991; Pipon, 1980, 1986), many of these genera have not gained acceptance by some European lichenologists (e.g., Clauzade and Roux, 1985; Eriksson and Hawksworth, 1986; Lima and Hladun, 2001; Poelt and Vézda, 1981; Purvis et al., 1992). More recently, some of these segregates have been combined on the basis of morphological and/or molecular characters (Blanco et al., 2004a, 2006; Elix, 1997, 2003; Hawksworth and Crespo, 2002). The generic concept within the clade including the large Hypotrachyna genus has not yet been reinvestigated, but molecular studies (e.g., Blanco et al., 2006) suggest that some of the proposed genera may not be monophyletic. The Hypotrachyna clade is an ideal model to evaluate the phylogenetic importance of vegetative thalline characters to circumscribe monophyletic groups, since here they have been widely employed in the distinction of genera (Table 1).

Parmeliaceae is one of the most common and speciose families of lichen-forming ascomycetes, including more than 2400 species classified in ca. 85 genera (Blanco et al., 2006; Hawksworth et al., 1995). The family is characterized morphologically by a certain type of ascoma development (Blanco et al., 2006; Elix, 1997, 2003; Hawksworth and Crespo, 2002). The generic concept within the clade including the large Hypotrachyna genus has not yet been reinvestigated, but molecular studies (e.g., Blanco et al., 2006) suggest that some of the proposed genera may not be monophyletic. The Hypotrachyna clade is an ideal model to evaluate the phylogenetic importance of vegetative thalline characters to circumscribe monophyletic groups, since here they have been widely employed in the distinction of genera (Table 1).

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In a previous phylogenetic study of foliose Parmeliaceae (Blanco et al., 2006) seven well-supported clades were found, the Hypotrachyna clade being one of them. Species of this clade are mostly tropical, and are characterized by a foliose, more or less dichotomously branched thallus, a pored epicortex, lack of pseudocyphellae, cell walls containing isochilenan, and bifusiform to cylindrical conidia.

Table 1

| Major characters used to circumscribe genera within the Hypotrachyna clade (Elix, 1993) |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Lobes | Subirregular to sublinear, irregularly to subdichotomously branched | Linear–elongate, regularly dichotomously branched, canaliculated | Linear–elongate, regularly dichotomously branched, canaliculated | Sublinear to linear–elongate, subdichotomously branched to dichotomously or irregularly branched | Subirregular, irregularly branched to dichotomously branched |
| Upper cortex | Palisade plectenchymatous | Paraplectenchymatous | Palisade plectenchymatous | Palisade plectenchymatous | Palisade plectenchymatous |
| Rhizines | Simple, sparsely or richly dichotomous | Simple to sparsely dichotomous | Simple to sparsely dichotomous | Richly dichotomous | Simple, in lobe axes |
| Marginal cilia | Bulbate | Simple or branched, evenly distributed | Simple or branched, evenly distributed | Absent or rarely present, then simple and in lobe axils | Simple, evenly distributed |
| Conidia | Cylindrical | Bifusiform | Bifusiform | Atranorin, lichenexanthone, usnic acid | Bifusiform |
| Cortical chemistry | Atranorin | Atranorin | Atranorin, lichenexanthone, usnic acid | Atranorin, secalonic acid A | Atranorin, secalonic acid A |
| Medullary chemistry | Orcinol depsides, orcinol depsides, beta-orcinol depsides, anthraquinones | Aliphatic acids | Orcinol depsides, orcinol depsides, beta-orcinol depsides, benzyl esters, aliphatic acids | Orcinol depsides, orcinol depsides, beta-orcinol depsides, beta-orcinol depsides, anthraquinones | Orcinol depsides, beta-orcinol depsides |


conidia. Some taxa of Parmelinopsis were placed in Hypotrachyna by Krog and Swinscow (1987). The generic circumscription of Everniastrum and Cetrariastrum has always been controversial (Culberson and Culberson, 1981; Sipman, 1980, 1986). Hale (1976a) described Everniastrum and Sipman (1980) segregated the genus Cetrariastrum from Everniastrum based on morphological differences, such as erect, shrubby thalli, irregularly dichotomously branched lobes, size of cilia, and some apothecial differences such as thickness of hypothecium. The two genera were later merged in Cetrariastrum by Culberson and Culberson (1981). They argued that the name Everniastrum was not validly published, which was confirmed by Serusiaux (1983). Sipman (1986) argued for a segregation of the two genera, validated the name Everniastrum and reiterated the differences that were interpreted as extremes of variation within the genus by Culberson and Culberson (1981). Bulbothrix is primarily a tropical–subtropical genus, characterized by bulbate marginal cilia, gray upper cortex, and cylindrical conidia and has its centre of distribution in South America and southern Africa (Hale, 1974a). The recently segregated genus Parmelinella (Elix and Hale, 1987) includes five species and is mainly distributed in South-east Asia. It accommodates taxa having moderately broad gray lobes, simple cilia, rhizines, and a yellow–gray upper cortex (containing secalonic acid A and atranorin).

Although various relationships within genera were discussed (Divakar and Upreti, 2005; Elix, 1994; Hale, 1975, 1976b), no overall phylogenetic hypothesis has been proposed. The backbone of the phylogenetic relationships within Parmelioid lichens is still uncertain and there is only weak support for the phylogenetic relationships within the Hypotrachyna clade (Blanco et al., 2006; Crespo and Cubero, 1998; Crespo et al., 2001). Further, the number of taxa studied were so far low and hence we performed a phylogenetic study focusing on species of the Hypotrachyna clade.

Previous phylogenetic studies of Parmeliaceae have shown that nuclear ITS rDNA and mitochondrial SSU rDNA were useful for assessing phylogenetic relationships at the generic level (e.g., Blanco et al., 2004a,b, 2005, 2006; Crespo and Cubero, 1998; Crespo et al., 2001; Mattsson et al., 2004; Thell et al., 2002). Therefore, we have chosen these two genes to reconstruct a phylogeny of Hypotrachyna and its allied genera. The resulting phylogenies are used to: (1) test the monophyly of genera described within the clade and (2) evaluate the phylogenetic value of morphological characters used to circumscribe Parmelioid genera in general.

2. Materials and methods

2.1. Taxon sampling

A total of 65 species, representing 52 of the Hypotrachyna clade, six of the related Parmelina clade, and seven belonging to other Parmelioid groups, were included in the analyses. Sequences of nu ITS rDNA and mt SSU rDNA were collected from 91 specimens. One hundred and twenty-one new sequences were obtained from 63 specimens and 55 were downloaded from GenBank (Table 2). Two species of the pseudocyphellate genus Parmelia s str. were used as outgroup, since the Parmelia clade has been shown to belong to a sister-group to the Hypotrachyna-clade previously (Blanco et al., 2006).

2.2. Molecular methods

Small samples prepared from freshly collected and frozen specimens were ground with sterile plastic pestles. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions but with slight modifications as described in Crespo et al. (2001). Dilutions of 1:10 of the total DNA were used for PCR amplifications of the nu ITS rDNA and mt SSU rDNA regions. Fungal nu ITS rDNA was amplified using the primers ITS1F (Gardes and Bruns, 1993), ITS4 (White et al., 1990), ITS1-LM (Myllys et al., 1999), and ITS2-KL (Lohhtander et al., 1998), while mt SSU rDNA was amplified using the primers msSSU1, msSSU3R (Zoller et al., 1999) and MSU1, MSU7 (Zhou and Stanosz, 2001). Amplifications were performed in 50 µl volumes containing a reaction mixture of 5 µl 10× DNA polymerase buffer (Biotools) (containing 2 mM MgCl₂, 10 mM Tris–HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, and 0.1% Triton X-100), 1 µl of dinucleotide triphosphate (dNTPs), containing 10 mM of each base, 2.5 µl of each primer (10 µM), 1.25 µl of DNA polymerase (1 U/µl), and 27.5 µl of H₂O. Finally, 40 µl of this mixture was added to 10 µl of DNA of each sample.

The amplifications for nu ITS rDNA were carried out in an automatic thermocycler (Techne Progene) and performed using the following programs: initial denaturation at 94 °C for 5 min and 30 cycles of: 94 °C for 1 min, 54–58 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The PCR amplification for mt SSU rDNA was carried out in a Hybaid OmniGene thermocycler and performed using the following program: initial denaturation at 94 °C for 5 min and 35 cycles of: 94 °C for 1 min, 55–58 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 5 min.

The PCR products were then cleaned using the Bioclean Columns kit (Biotools) according to the manufacturer’s instructions. The cleaned PCR products were sequenced using the same primers as used for PCR amplifications. The ABI Prism™ Dye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) was used and the following settings were applied: denaturation for 3 min at 94 °C, 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequencing reactions were electrophorezed on a 3730 DNA analyzer (Applied Biosystems). Partial nu SSU rDNA, including an intron at the end of the 3′ (SSU), were removed before the alignment. Sequence fragments obtained were assembled with SeqMan 4.03 (DNASTAR) and manually adjusted.
Table 2
Specimens used in the study, with location, reference collection detail, and GenBank accession numbers

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<th>Collector(s)</th>
<th>Voucher specimens</th>
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<th>GenBank no. mtSSU</th>
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</table>

(continued on next page)
2.3. Sequence alignments

We used an alignment procedure employing a linear Hidden Markov Model (HMM) for the alignment, as implemented in the software SAM (Hughey and Krogan, 1996). Sequences of 91 specimens (Table 2) were separately aligned for the two genes. Regions that could not be aligned with statistical confidence were excluded from the phylogenetic analysis. A small region that remained ambiguously aligned in the nu ITS and mt SSU rDNA matrices was excluded before the analyses.

2.4. Phylogenetic analyses

The alignments were analyzed by MP and a Bayesian approach (B/MCMC). The program MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001) was employed to sample trees using a Markov Chain Monte Carlo (MCMC) method. The analyses were performed assuming the general time reversible model of nucleotide substitution (Rodriguez et al., 1990), including estimation of invariant sites, assuming a discrete gamma distribution with six rate categories and allowing site specific rates (GTR + I + G + SS) by using the covariation (Tuffley and Steel, 1998) option of MrBayes for the single gene and the combined analyses. No molecular clock was assumed. Parallel runs were made with 2,000,000 generations starting with a random tree and employing four simultaneous chains each. Every 100th tree was saved into a file. The first 200,000 generations (i.e., 2000 trees) were deleted as the “burn in” of the chains.

We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) to ensure that stationarity was achieved after the first 200,000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck and Ronquist, 2001). Of the remaining 18,000 trees a 50%-majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Posterior probabilities equal to and above 95% were considered strong supports.
analyses, the set of topologies reaching 0.95 posterior probability was estimated. The combined analysis topology was then compared for conflict with the 0.95 posterior intervals of the single gene analyses. If no conflict was evident, it was assumed that the two data sets were congruent and could be combined. If conflict was evident, the two data sets were interpreted as incongruent and thus the combined analysis could be potentially misleading (Bull et al., 1993).

A maximum parsimony (MP) analysis was performed using PAUP 4.0b10 (Swofford, 2003). A heuristic search of 100 simple taxon addition replicates was conducted with TBR branch-swapping and the MulTrees option in effect, equally weighted characters, and gaps treated as missing data. Separate analyses of the two data sets were performed. Since no hard conflict (supported by at least 75% bootstrap support) was evident, it was assumed that data sets were congruent and hence a combined analysis was performed. Non-parametric bootstrap (Felsenstein, 1985) was used to assess robustness of clades, running 1000 pseudoreplicates with the same settings as in the heuristic search. Only clades that received bootstrap support equal or above 75% were considered as strongly supported. Phylogenetic trees were drawn using TREEVIEW (Page, 1996).

Since the results of the phylogenetic analyses were incongruent with the current classification of genera in the Hypotrachyna clade, we tested whether our data were sufficient to reject the following three alternative hypotheses: (1) monophyly of Hypotrachyna, (2) monophyly of Cetrariastrum+Everniastrum, and (3) monophyly of Bulbothrix. Such topologies may not be significantly worse than the obtained topology. For the hypothesis testing three different methods were employed: (1) Bayesian hypothesis testing following Ihlen and Ekman (2002) and Lumbsch et al. (2004), (2) the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), and (3) expected likelihood weight (ELW) test following Strimmer and Rambaut (2002). For the Bayesian hypothesis testing a run of 1 Mio. generations was performed with the same settings as in the estimation of the phylogeny using the combined data set. Five thousand trees at the equilibrium state for the null hypothesis were used from this analysis. The probability of the null hypothesis being correct was calculated by counting the presence of this topology in the MCMC sample (Lewis, 2001). The frequency of trees in the MCMC sample agreeing with the null hypothesis was calculated using the filter command in PAUP* with a constraint describing the null hypothesis. The SH and ELW tests were performed using Tree-PUZZLE 5.2 (Schmidt et al., 2002), the best trees agreeing with the null hypotheses, and the unconstrained ML tree. These trees were inferred in Tree-PUZZLE employing the GTR+I+G nucleotide substitution model.

3. Results

3.1. Phylogenetic analyses

A total of 62 new mitochondrial SSU rDNA and 59 new nuclear ITS rDNA sequences were generated (Table 2). Fifty-five sequences were downloaded from GenBank (Table 2) and aligned with the former ones. The aligned matrix contained 793 unambiguously nucleotide position characters in mt SSU and 463 in nu ITS. The final alignment of combined data set was 1256 positions in length with 525 variable characters.

A parsimony analysis of combined data matrix resulted in 505 equally most parsimonious trees (tree length = 2463 steps, CI = 0.34, and RI = 0.77). Eighty-eight positions in the matrix were parsimony-uninformative and 437 informative. In the B/MCMC analysis, the likelihood parameters in the sample had the following mean (Variance): lnL = -1345.36 (1.4), base frequencies \( \pi(A) = 0.298 \) (0.0003), \( \pi(C) = 0.201 \) (0.0002), \( \pi(G) = 0.2 \) (0.0003), \( \pi(T) = 0.301 \) (0.0004), rate matrix \( r(AC) = 0.745 \) (0.019), \( r(AG) = 0.24 \) (0.0006), \( r(AT) = 0.109 \) (0.005), \( r(CG) = 0.666 \) (0.003), \( r(CT) = 0.436 \) (0.008), \( r(CT) = 0.734 \) (0.002), the gamma shape parameter \( \alpha = 0.439 \) (0.004) and the proportions of state (off → on) = 0.341 (0.005), state (on → off) = 0.525 (0.089), and proportion of invariant sites \( p(\text{invar}) = 0.593 \) (0.007).

Since the overall topologies of the MP and B/MCMC analyses were identical, only the 50% majority-rule consensus tree of 18,000 sampled trees in the B/MCMC analysis is shown with MP bootstrap values added (Fig. 1). B/MCMC posterior probabilities equal or above 0.95 are indicated by bold branches and MP bootstrap values above 74% are indicated at branches.

In the majority-rule consensus tree of combined data set shown in Fig. 1, the taxa of the Hypotrachyna clade fell into two distinct and well-supported groups. Group I including Hypotrachyna sensu stricto (type species H. brasiliiana), Cetrariastrum, Everniastrum, and Parmelinopsis species, the latter two nested within Hypotrachyna. Group II includes mostly Asian species of Hypotrachyna and species of Bulbothrix and Parmelinella. In total eight well-supported clades within the Hypotrachyna clade can be distinguished, four of them containing taxa currently referred to Hypotrachyna: (1) a strongly supported Parmelinopsis–Hypotrachyna clade (clade A) containing all included species of Parmelinopsis and four species of Hypotrachyna; (2) a clade with six Hypotrachyna species, including the type (H. brasiliiana) (clade B); (3) a clade with ca. eight Hypotrachyna taxa (clade D); and (4) an Asian-Hypotrachyna clade, containing nine south-east Asian species and one in a basal position from South America (clade E).

Other resolved clades of parmelioid lichens included are: a strongly supported Everniastrum clade (clade C), a Bulbothrix p.p. clade (clade G), a Parmelinella–Bulbothrix clade (clade F), containing the type species of Parmelinella and two Bulbothrix species, and the Myelochroa clade (clade H), which also contains Hypotrachyna radiculata. Clade A–D, Cetrariastrum ecuadorense and Hypotrachyna fiscarcarpa form group I. Group II consists of clades E–G, while H. radiculata falls outside the Hypotrachyna clade and clusters within Myelochroa.

The results of the three tests for probabilities of alternative topologies are shown in Table 3. Monophyly of Bulbo-
thrix and Hypotrachyna are significantly rejected by all tests, while the tests fail to reject a topology that has Cetrariastrum + Everniastrum in a monophyletic clade.

3.2. Phylogenetic pattern of morphological characters

The phylogenetic occurrence of selected morphological characters that are widely used to distinguish genera in Parmelioid lichens and that have been important in generic circumscriptions in the Hypotrachyna clade are plotted onto the phylogenetic tree in Fig. 2. Two types of upper cortices occur within the Hypotrachyna clade, being a palisade plectenchyma or paraplectenchymatous. While all taxa of group II have a palisade plectenchyma clade, both types occur within group I. Taxa currently classified in Cetrariastrum and Everniastrum have a
paraplectenchymatous cortex, while a palisade paraplectenchyma occurs in *Hypotrachyna* clade A, which includes *Hypotrachyna* and *Parmelinopsis* species. The characters cilia and rhizines are more homoplasious. Group I includes species with simple or branched cilia or taxa lacking cilia and all these character states appear to have originated at least twice within this clade.

In group II, the phylogeny also suggests parallel evolution of character states absence and simple cilia, while bulbate cilia may have been evolved only once within this clade, but are also known from *Relicina*, which does not belong to the *Hypotrachyna* clade. Rhizines are either lacking, simple, sparsely dichotomous, or richly dichotomously branched in group I. With the exception of simple
rhizines, all these character states appear to have evolved in this group more than once given the inferred phylogeny. In group II, only species with richly dichotomous and simple rhizines occur, and the latter only occur in a well-supported clade including three *Bulbothrix* species and *Parmelinella*. In group I, lobes may be sublinear or linear–elongate, both character states occur in two clades. Sublinear and subirregular lobes occur in group II, both characters showing no homoplasies.

4. **Discussion**

4.1. **Phylogenetic relationships**

The species of the *Hypotrachyna* clade mainly fall into two major groups that are strongly supported. Most of the genera belong to either of the major groups with the exception of *Hypotrachyna*, which occurs in both major groups and also outside the *Hypotrachyna* clade. Some tendencies of morphological differences between species of group I and II can be seen, but none of these characters appears to be consistent. Species of group I have for example narrow, sublinear–elongate lobes with truncate apices. Although lobes of *Bulbothrix* are generally subirregular rotund, sublinear–elongate lobes also occurs in some species (Hale, 1976c), such as *B. apophysata*, *B. coronata*, or *B. goebellii* (Clade G).

Species of group I contain orcinol depsides, such as glyphoric acid, beta-orcinol depsides (e.g., barbatic acid), orcinol depsidones, such as livicid acid, beta-orcinol depsidones (e.g., protocetraric acid), aliphatic acids, such as protolichesterinic acid, pigments (e.g., pigmentosin A, skyrin), benzyl esters (e.g., alectorialic acid), dibenzofuranes, such as usnic acid, and the non-chlorinated licheanthone. The taxa in group II possess as secondary metabolites orcinol depsides, such as glyphoric acid, beta-orcinol depsides (e.g., atranorin), orcinol depsidones, such as lobaric acid, beta-orcinol depsidones (e.g., salazinic acid), aliphatic acids, such as caperatic acid, pigments (e.g., skyrin), and bis-xanthones (e.g., secalonic acid A).

The distribution of the genera in the *Hypotrachyna*-clade has been discussed in several papers (Culberson and Culberson, 1981; Elix and Hale, 1987; Hale, 1975) and a neotropical centre of distribution for most of these genera has been proposed. Species in clade A have mostly a paleotropical distribution, while clade B, C, and D include pantropical and neotropical species. In temperate regions, the species are restricted to coastal areas but in the tropics they usually occur in high or moderately high elevations. The group II is pantropical with two distinct centres of distribution, in south-east Asia for the *Hypotrachyna* clade E (Divakar and Upreti, 2005), and in the tropical montane regions of the Neotropics and tropical Asia for *Bulbothrix* species (Hale, 1975; Kurokawa and Lai, 2001; Louwhoff and Elix, 2002). Clades F and G include taxa occurring in montane forests of the Paleo- and Neotropics.

Our extended taxon sampling confirmed our previous results (Blanco et al., 2006) that *Hypotrachyna* as currently circumscribed is polyphyletic. Also the two genera *Bulbothrix* and *Parmelinopsis* are not monophyletic, while *Everniastrum* and *Parmelinella* are monophyletic, but nested within *Hypotrachyna* and *Bulbothrix*, respectively. This clearly shows that the generic concept within the *Hypotrachyna* clade requires revision.

The generic distinction of *Cetrariastrum* and *Everniastrum* has been disputed (Culberson and Culberson, 1981; Sipman, 1980, 1986), but in our analyses the type species of *Cetrariastrum*, *Cetrariastrum ecuadorense*, does not cluster within the *Everniastrum* clade. Since an alternative topology of a monophyletic *Cetrariastrum + Everniastrum* cannot be rejected, the question remains open. Denser taxon sampling in this clade and especially addition of further *Cetrariastrum* species is necessary to resolve the phylogenetic relationships of the two genera.

Elix and Hale (1987) described *Parmelinopsis* and separated it from *Hypotrachyna* based on conidial characters, and morphological traits, such as cilia- and rhizine-type. However, the two genera are morphologically very similar in having narrow, sublinear, subdichotomously branched lobes with truncate apices and share a pantropical distribution. Our molecular analysis suggests that these similarities are due to a common origin and that *Parmelinopsis* should be synonymized with *Hypotrachyna* (*H. brasiliiana*, the type species, clusters in clade B).

The genus *Bulbothrix* is characterized by having bulbate marginal cilia and a gray upper cortex, but fell into two monophyletic clades: Clade F (South African and tropical Asian lineage), which includes *Parmelinella wallichiana* (a non-bulbate ciliate genus) and Clade G (South African and South American lineage).

Surprisingly, *H. radiculata* falls well supported within *Myelochroa* (Clade H) outside the *Hypotrachyna* clade. This species has been placed in *Parmelinopsis* (Elix and Hale, 1987) before Elix (2001) transferred it into *Hypotrachyna*. It is somewhat aberrant within *Hypotrachyna sensu lato* in containing triterpenes, which only occur in the closely related *H. majoris*, although terpenes are common in *Myelochroa*.

Our study supports that the genus *Relicina*, which is morphologically similar to *Bulbothrix*, but differs in having usnic acid as cortical chemistry and bifusiform conidia, and does not belong to the *Hypotrachyna* clade. Since *Relicina* species share most morphological characters with *Bulbothrix* species and differ in characters that occur in other taxa of the *Hypotrachyna* clade, this and the placement of *H. radiculata* raises the question of a morphological definition of the *Hypotrachyna* clade. Currently, we are not aware of any distinct morphological or chemical character that would circumscribe the *Hypotrachyna* clade, but spore cell walls and other ascomatal characters are currently being studied using transmission and scanning electron microscopy.

4.2. **Phylogenetic pattern of morphological characters**

Morphological characters currently used in parmelioid lichens to circumscribe genera are homoplasious within the
Hypotrachyna clade. None of these characters correspond to the two major and well-separated groups found within the Hypotrachyna clade. This is especially true for cilia and rhi- zines that have been widely used to distinguish genera within parmelioid lichens. Furthermore, Divakar et al. (2005) demonstrated that the rhi- zines are useful characters at specific level in Parmelia. This demonstrates that appen- dicular structures of the main thallus have been overesti- mated in previous classifications, as has also been demonstrated for parmotremoid lichens (Blanco et al., 2005). Surprisingly, lobe shape seems to be less homoplas- ious within the Hypotrachyna clade, although it cannot dis- tinguish the two major groups within the clade. Cortical anatomy may also only be used as an additional feature in a forthcoming new generic classification within the Hypo- trachyna clade. Although the character is uniform within group II, group I exhibits both character states and thus there is no definite distinction of both groups possible on the basis of these features. In the absence of any known morphological character that correlates well with the molecularly inferred phylogeny, a revised generic classification within this clade must await a thorough re-examina- tion of morphology and anatomy within this clade.

Acknowledgments

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