Phylogeny of the Acarosporaceae (Lecanoromycetes, Ascomycota, Fungi) and the evolution of carbonized ascomata

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Abstract. The phylogeny of the Acarosporaceae (Lecanoromycetes, Acarosporomycetidae, Acarosporales) is investigated using data from three molecular markers; nuclear ITS-LSU rDNA, mitochondrial SSU and β-tubulin. Acarosporaceae is shown to be constituted by six main clades; *Myriospora*, *Timdalia, Pleopsisium*, a clade composed by “*Acarospora*” *rhizobola* and “*A.*’ *terricola*, the poorly supported *Sarcogyne* clade (including several *Polysporina* and *Acarospora* species) and the *Acarospora* clade (including the type of *Polysporina*, *P. simplex*, and several other *Polysporina* species). The common ancestor of the Acarosporaceae did not produce strongly black pigmented (carbonized or melanized) ascomata, but this trait has arisen secondarily and independently numerous times in the evolution of the group. The number of changes in character states of both carbonized epihymenium and carbonized exciple are considerably more than the minimum number. The genera *Sarcogyne* and *Polysporina* – largely circumscribed based on the presence of black pigmented ascomata – are shown to be distinctly non-monophyletic. The presence of green algae in the ascoma margin (lecanorine or lecideine ascomata) may vary even within single species.

Keywords: Convergent evolution, lichens, lichenized fungi, lichenicolous
Carbonization or melanization in fungal structures is the accumulation of various degrees of a black pigmentation, which generally is presumed to consist of melanins. Melanins are widely present in fungi and may have several functions, including reducing the damaging effects of solar radiation (Butler & Day 1998), and it is widely assumed that they have this protective function also in lichens (Rikkinen 1995; Gauslaa & Solhaug 2001; Hauck et al. 2007). Lichen mycobionts produce various types of melanins with different metabolic pathways (Rikkinen 1995).

Melanin made from 1,8 dihydroxynaphtalene is a synapomorphy for the Euascomycetes/Pezizomycotina (Tehler 1988), including most lichen mycobionts. Little is known, however, about the molecular background and the observed intraspecific variation often seen in the production of melanin (e.g. Nybakken et al. 2004, Rikkinen 1995). As an obvious and easy-to-recognize characteristic, carbonization of different fungal fruiting body structures has been used as an important character for generic circumscription in some taxonomic groups, for instance the Acarosporaceae (Flotow 1851; Magnusson 1935; Vězda 1978). When these genera are accepted in the current classifications, this implicitly assumes that the presence of this trait characterizes natural, monophyletic groups. Understanding the evolution of carbonized structures is thus crucial for our understanding of the evolution of this and other fungal groups and for how we express the phylogenetic relationships in our classifications.

Acarosporaceae is a distinct group of predominantly crustose lichenized fungi, growing on exposed rocks and soil on all continents. The members are primarily characterized by the multi-spored ascus (mostly >100 small, simple, colourless
spores), and they were among the earliest lichenized groups to appear (the split from the rest of the Lecanoromycetes appeared in late Carboniferous-early Permian but among the most recent groups to diverge (upper Cretaceous; Prieto & Wedin 2013). Originally (Zahlbruckner 1907) Acarosporaceae included five genera (Acarospora, Biatorella, Thelocarpon, Maronea and Glypholecia). The number of genera successively increased (Magnusson 1935, Poelt 1974, Eriksson & Hawksworth 1991), although it was noted that the family was heterogenous (Poelt & Vězda 1981). Hafellner (1992, 1993, 1995) re-assessed the family, and suggested a new family concept based on a shared ascus-type without amyloid structures and a non-amyloid or weakly amyloid tholus. He excluded the re-instated Pleopsidium from the family on account of its amyloid tholus. The circumscription of Acarosporaceae and the relationship to Lecanorales and Lecanoromycetes has since been investigated several times using molecular phylogenies. Stenroos and DePriest (1998) were first to indicate that Acarosporaceae grouped outside Lecanorales s. str., and several later investigations suggested that the family was a phylogenetically distinct group that formed the sister-group to a large part of the Lecanoromycetes (Reeb et al 2004, Wedin et al 2005, Schoch et al 2009, Bendiksby and Timdal 2013). Reeb et al. (2004) excluded the family from Lecanorales and described the subclass Acarosporomycetidae which then included Acarospora, Glypholecia, Pleopsis, Polysporina, Sarcogyne and Thelocarpella. Wedin et al. (2005) confirmed the position of Pleopsis and in addition showed that Timdalia, a recent segregate from Acarospora (Hafellner & Türk 2001) with a similar ascus-type as in Pleopsis, also belong in a well-supported monophyletic Acarosporomycetidae/Acarosporaceae. Finally Crewe et al. (2006), Wedin et al. (2009), and Westberg et al. (2011) suggested that the Acarospora smaragdula group...
formed the sister-group to the rest of Acarosporaceae. This group was formally
recognized as the genus *Silobia* (Westberg et al. 2010), a name that was replaced by
the older name *Myriospora* (Arcadia & Knudsen 2012). The monophyletic
Acarosporaceae currently contains eight genera confirmed by molecular
phylogenies; *Acarospora, Glypholecia, Myriospora, Pleopsidium, Polysporina, Sarcogyne, Thelocarpella* and *Timdalia*. Two further genera, *Caeruleum* and
*Lithoglypha*, are presumed to belong within Acarosporaceae, but this has so far not
been confirmed with molecular data. A recent analysis has also indicated that the
genus *Eiglera* is closely related to Acarosporaceae and if this is confirmed
Eigleraceae should be included in the Acarosporomycetidae (Miadlikowska et al.
2014).

In the Acarosporaceae, *Acarospora, Myriospora, Pleopsidium* and *Timdalia* have
ascomata with margins that contain green algae (the apothecia are referred to as
lecanorine, pseudolecanorine, or more often cryptolecanorine). Three genera,
*Lithoglypha, Polysporina*, and *Sarcogyne*, are characterized by algal-free lecideine
ascomata often with carbonized margins and/or discs (Fig. 1). The carbonization has
been fundamental for the current circumscription and delimitation of *Sarcogyne* and
*Polysporina* (Knudsen 2007b, Knudsen and Standley 2007).

*Sarcogyne* (Flotow 1851) was originally described for *S. corrugata* (=*S. clavus*)
a species with lecideine apothecia with very strongly carbonized margin (Fig. 1).
Magnusson (1935) expanded the concept of the genus and included a large number
of species with lecideine apothecia with or without a strongly carbonized margin.
Věžda (1978) later separated a number of species primarily characterized by a thick,
fissured, carbonized margin and a carbonized epihymenium (typically forming
umbonate apothecia) but also with richly branched and anastomosing paraphyses.
with non-swollen apices, into *Polysporina* (Fig. 1). *Sarcogyne* in Vězda’s restricted sense thus had a non-carbonized epihymenium and stouter, poorly branched paraphyses with swollen apices while the margin either was strongly carbonized (as in *S. clavus* where the interior of the margin is black pigmented and friable throughout) or not (as in *S. regularis* which is only black pigmented at the surface) and there are also examples of species with intermediary levels of carbonization as in *S. similis* (the margin is black throughout but tar-like in texture, Knudsen and Etayo 2009). Later authors, however, also included species with stout, simple paraphyses in *Polysporina* (Ahti et al. 1987, Kantvilas 1998, Kantvilas & Seppelt 2006) and the only difference between the two genera at present is the carbonized epihymenium (a build–up of carbonized accretions on the apothecial surface) in *Polysporina*. The distinction between the genera is thus not clear and in need of re-assessment (Hafellner 1995, Kantvilas and Seppelt 2006).

In this paper we investigate the phylogeny of the Acarosporomycetidae/Acarosporaceae with the specific aim to clarify the evolution of carbonized/melanized ascomata, with and without a carbonized epihymenium, in this group. These traits are currently considered to characterize *Sarcogyne* and *Polysporina*, and the monophyly of these two genera will be tested here.
Material & Methods

Molecular data

Taxon sampling

Species selected for sequencing were sampled broadly to cover as many different genera and morphological groups as possible. We aimed at including two specimens from different localities of each taxon. We obtained molecular data for 128 specimens (Suppl. Tab. 1 representing c. 62 taxa. The ingroup comprised 127 specimens, which included 57 representatives of Acarospora, 1 of Glypholecia, 11 of Myriospora, 3 of Pleopsisdium, 36 of Polysporina, 17 of Sarcogyne, and 2 of Timdalia. In spite of several attempts on fresh material we were not successful in sequencing Caeruleum heppii. Fresh material of Lithoglypha and Thelocarpella was not available to us in this study. Pycnora sorophora was used as outgroup.

DNA extractions, amplification, and sequencing

DNA was extracted from recently collected field material or from dried herbarium specimens (Suppl. table 1). Total DNA was extracted using the Qiagen DNeasy Plant MiniKit, according to the manufacturer’s instructions.

The selected markers for this study were the internal transcribed spacer complete repeat (ITS) and the large subunit (nLSU) of the nuclear ribosomal DNA, the small subunit of the mitochondrial ribosomal DNA (mtSSU), and the coding sequence of the β-tubulin gene. A fragment of ca. 696 bp in the β-tubulin marker was amplified using the primers BT3LM5 and BT10LM3 (Myllys et al., 2001) or with primers newly designed in this study (see Suppl. table 2). The primers ITS1F (Gardes and
Bruns, 1993) and LR3 (http://www.biology.duke.edu/fungi/mycolab/primers.htm) were used to amplify the internal transcribed spacer I, the 5.8 rDNA gene, the internal transcribed spacer II and a fragment of approximately 600 bp in the nLSU rDNA gene. The mtSSU was amplified using the primers mrSSU1 and mrSSU3R (Zoller et al., 1999).

PCR amplifications were performed using Illustra™ Ready-To-Go PCR Beads, according to the manufacturer’s instructions, with the following settings for ITS, nLSU and mtSSU rDNA: initial denaturation 94°C for 5 min, followed by five cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s), and finally 30 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 60 s), with a final extension 72°C for 300 s; and with the following settings for the gene coding for β-tubulin: initial denaturation 94°C for 5 min, followed by five cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 60 s) and finally 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s), with a final extension 72°C for 300 s.

Before sequencing, the PCR products were purified using the PCR-M® Clean-up System of Viogene or the enzymatic method Exo-sap-IT© provided by USB Corporation.

The PCR-products were sequenced and purified using the DYEnamic ET terminator cycle sequencing kit protocols (Amersham Biosciences, Freiburg, Germany), with the following settings: 25 cycles (95°C for 20 s, 50°C for 15 s, and 60°C for 60 s).

The ITS rDNA sequences were produced using the PCR primers as above, plus the additional primer ITS4 (White et al., 1990) when necessary. The mtSSU rDNA and
the β-tubulin sequences were produced using the PCR primers plus additional internal primers (Suppl. Tab. 2) for β-tubulin when necessary. The purified samples were run on an automated sequencer (ABI Prism 377).

Sequence alignment and phylogenetic analyses
Sequences were aligned using the multiple sequence alignment software MAFFT version 7.110 (Katoh et al., 2002, Katoh and Toh 2008a). The G-INS-i algorithm was used for the β-tubulin sequences and the Q-INS-i for the ITS and nLSU sequences (Katoh and Toh 2008b). β-tubulin sequences were translated to amino acids, and three identified introns were manually removed from the alignment.

Major insertions and ambiguous regions in the ITS and nLSU alignments were identified and eliminated with Gblocks version 0.91b (Castresana, 2000) using the relaxed parameter values suggested by Talavera and Castresana (2007).

We assessed congruence analysing the datasets separately by ML bootstrapping, to detect possible conflicts among clades. Conflict was understood as bootstrap support (≥ 70%; Hillis and Bull, 1993) for one marker, contradicted with significant support by another. No incongruence was found and the data were concatenated into a single dataset.

We used Maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) for the phylogenetic analyses using the combined dataset. Maximum parsimony and parsimony bootstrap analyses were performed using TNT 1.1 1 (Tree analysis using New Technology, Goloboff et. al, 2008). We did heuristic searches (‘traditional search’) collapsing ‘rule 3’ (tree collapsing = max. length 0; collapsing branches with no possible support), 1000 random addition sequence replications and holding up to 1000 trees during each replication, using a tree
bisection and reconnection (TBR) swapping algorithm. If the most parsimonious
trees (MPT) were found in only a few replications we broadened the search to
include more replications and holding more trees per replication. We also
performed a total of 1000 bootstrap replicates with the same specifications but with
100 random-addition sequence replicates. Parsimony-uninformative characters were
excluded from these analyses.

We performed ML analyses in RAxMLGUI 1.3, a graphical front-end for
RAxML (Randomized Accelerated Maximum Likelihood for High Performance
Computing; Stamatakis, 2006), using the GTRCAT model of nucleotide substitution
(a GTRGAMMA approximation with optimization of individual per-site
substitution rates). We partitioned the dataset by gene and by codon position in the
protein-coding gene (β-tubulin), which made a total of 8 partitions (ITS1, 5.8S,
ITS2, nLSU, mtSSU, BT 1st, BT 2nd and BT 3rd). The same model was applied to all
partitions because of constraints of the software RAxML. We performed a total of
100 runs and assessed node support via 1000 bootstrap replicates (ML + thorough
bootstrap; n. threads 2).

Bayesian analysis (Huelsenbeck et al., 2001) was achieved with the software
MrBayes 3.2.1 (Ronquist et al. 2011). We partitioned the dataset as in the ML
analyses, but in this case we selected the model of nucleotide substitution that
scored best for every particular partition, according to the Akaike Information
Criterion (AIC) in jModeltest (Posada, 2008). We used full likelihood optimization
and searched only among the 24 models implemented in MrBayes. Following this
scheme, a HKY model was selected for ITS1 and ITS2, a HKY+I+Γ model was
selected for the 5.8S and for the mitochondrial SSU DNA, a SYM+I+Γ model was
selected for the nuclear LSU rDNA, and a GTR+I+Γ, a SYM+I+Γ, and a SYM+Γ
models were selected for the 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} codon positions of the β-tubulin gene, respectively. We linked topology across partitions but separated model parameter values and proportional rates across partitions. The number of discrete gamma categories was kept at default four. Bayesian prior distributions included treating all tree topologies as equally likely, a uniform (0, 50) distribution for the gamma shape parameter, a uniform (0, 1) distribution for the proportion of invariable sites, a flat (1, 1, 1, 1, 1, 1) Dirichlet for the rate matrix, and a flat (1,1,1,1) Dirichlet for the state frequencies (except when the model dictated state frequencies to be equal). We performed three parallel runs, each with five chains, four of which were incrementally heated with a temperature of 0.10. The analysis was diagnosed for convergence every 100000 generations, measured as the average standard deviation of splits across runs in the last half of the analysis. Every 100th tree was saved. The first half of the run was discarded as burnin.

\textit{Hypothesis testing}

We used Bayes factors to test the monophyly of \textit{Acarospora}, \textit{Polysporina} and \textit{Sarcogyne}, and also of the core group of \textit{Sarcogyne}, i.e., those species that, like the type species, \textit{S. clavus}, have a strongly carbonized excipulum (e.g., Knudsen & Kocourková 2008), in this analysis, \textit{S. algoviae}, \textit{S. clavus}, \textit{S. hypophaea}, \textit{S. hypophaeoides} and \textit{Sarcogyne} sp. 1. To calculate the Bayes factors we compared the ratio of the marginal likelihoods of five hypotheses: four where each group was constrained to be monophyletic, in addition to the best polygenetic hypothesis recovered by MrBayes 3.2.1 (Ronquist et al., 2011) in the unconstrained analysis. The Bayes factors were calculated using the modification introduced by Kass and
Raftery (1995), i.e. twice the difference between the ln harmonic mean likelihoods of the two models.

Reconstruction of ancestral character states

We reconstructed the ancestral states of two characters, the carbonized exciple and the carbonized epiphymenium. The first character, carbonized exciple, has three states, i.e., absence of carbonized exciple (0), presence of carbonized exciple (1), and tar-like exciple (2). The second is a binary character coded as absence (0) or presence (1) of carbonized epiphymenium. The coding follows our own observations.

For the reconstruction of ancestral states we used maximum parsimony and maximum likelihood as implemented in Mesquite v. 2.75 (Maddison and Maddison, 2011) and two Bayesian approaches, i.e., the method described by Huelsenbeck and Bollback (2001) as implemented in SIMMAP v.1.5 (Bollback, 2006), following Schultz and Churchil (1999), and the method described by Pagel et al. (2004) and Pagel and Meade (2006), as implemented in BayesTraits (Pagel and Meade, 2007). For all four methods, we used the posterior tree sample from the MrBayes analyses. Branch lengths from that analysis were included in the ML and in the Bayesian reconstructions. Maximum parsimony and maximum likelihood reconstructions were achieved counting only unequivocal states. ML reconstructions were performed under the Mk1 likelihood model for discrete morphological characters (Lewis, 2001). For the SIMMAP analyses, we estimated three prior parameters: the shape parameter \( \alpha \), and the scale parameter \( \beta \), for the gamma distribution of the overall substitution rate, and the shape parameter \( \alpha \) for the beta distribution of the bias parameter, this last one required only for binary morphological characters (Bollback, 2006). The estimation was achieved in two steps following SIMMAP
documentation on morphology priors, and using the R package provided by SIMMAP v. 1.5 (Bollback, 2006). Following this approach, the parameter values for the gamma distribution of the overall substitution rate were $\alpha = 11.728$ and $\beta = 3.784$ for character 1 (carbonization of the exciple), and $\alpha = 4.248$ and $\beta = 1.550$ for character 2 (carbonization of the hymenium). The single parameter value for the beta distribution of the bias parameter in character 2 was $\alpha = 3.778$. The number of discrete gamma categories was kept at default 50. The morphological tree was rescaled to a total length of 1. In the BayesTraits analyses, the prior on rates was assumed to follow an exponential distribution with the mean drawn from a uniform hyperprior on the interval $(0, 10)$. The MCMC was run for $10^8$ generations, sampling parameters every 1000th generation, and discarding the first $10^7$ generations as burn-in. The rate deviation of the normal distribution was set so that the MCMC acceptance rate was between 20% and 40%. Each analysis was conducted three times and similar harmonic mean likelihoods obtained across identical runs indicated that MCMC chain had converged. To extract the marginal posterior probability of each state at each node (integrated over priors, tree topologies and branch lengths) from BayesTraits output, we used software written by S. Ekman (Ekman et al., 2008).

In addition, we obtained the distribution of the number of character state transformations along the Acarosporaceae using a) Maximum parsimony as implemented in Mesquite (Maddison and Maddison 2011) and b) the Bayesian stochastic mapping procedure described by Huelsenbeck et al. (2003) and Ronquist (2004), as implemented in SIMMAP (Bollback, 2006). We used the same Bayesian tree sample and the same rate priors as in the ancestral state reconstruction using.
SIMMAP. The number of realizations from the prior distribution was set to 10 per tree.
Results

We produced 112 new ITS rDNA, 126 new nLSU rDNA, 111 new mtSSU rDNA, and 109 new β-tubulin sequences, and additional sequences (all produced in our earlier work) were used from GenBank (Suppl. Tab. 1). We produced a combined matrix of the four markers, which included a total of 512 sequences. Of the 128 terminals, only 5 lack β-tubulin- Therefore, sequences for all three markers were available for 96% of the terminals. The combined matrix included (Suppl. Tab. 3) a total of 2446 characters of aligned DNA sequences from mitochondrial and nuclear genes: ITS1 rDNA (122 bp), 5.8S rDNA (117 bp), ITS2 rDNA (175 bp), nLSU rDNA (584 bp), mtSSU rDNA (752 bp), and β-tubulin (696 bp).

The MP analysis of the combined dataset resulted in 124 most parsimonious trees of 4798 steps. The best tree obtained from the ML analysis had a ln-likelihood value of -24236.56. The Bayesian analyses of the combined dataset halted after 900,000 generations, at which time the average standard deviation of splits across runs in the last half of the analysis was 0.009 (<0.01). Potential Scale Reduction Factor (PSRF) values for all model parameters as well as all branch lengths were close to 1 (none of the PSRF values of the model parameters was over 1.004). We considered the three runs to have converged and that our sample was a valid estimate of the posterior distribution. A majority rule consensus tree was constructed from the 13,500 trees of the stationary tree sample. As the phylogenetic reconstructions obtained by the three inference methods (MP, ML and BI) were congruent, only the topology corresponding to the Bayesian analyses is shown in Fig. 2.

The analyses find a number of strongly supported clades (Fig. 2) but several branches in the backbone of the tree lack support or have support only from BI.
Myriospora form a monophyletic group as the sister-group to the rest of the family. The sampled species in Pleopsidium, Timdalia and a clade formed by “Acarospora” terricola and “A.” rhizobola also form distinct groups well separated from other genera. The remaining species roughly form two clades, a well-supported *Acarospora* clade including the type *A. schleicheri* and a clade dominated by *Sarcogyne* species. The *Acarospora* clade is further composed of a well-supported main clade of *Acarospora* and *Polysporina* species, the sister group of which is a small clade formed by three *Polysporina* representatives, although this relationship is only supported in the Bayesian analysis. The clade dominated by *Sarcogyne* species has no support in any of the analyses, but the group formed by *Acarospora* and *Sarcogyne* is supported by all three methods (Fig. 2). The overall support of the phylogeny is high: 81% of the nodes are supported by the Bayesian analysis, 73% of the nodes are supported both by Bayesian PPs and ML bootstrap, and 63% were supported by all three methods (Bayesian PPs, ML, and MP bootstraps). Ten nodes are supported by PPs only, including three nodes in the backbone of the phylogeny (Fig. 2).

**Hypothesis testing**

The position of a few taxa such as *Acarospora macrospora* or *Sarcogyne hypophaeoides* is unstable, and all hypotheses considering *Acarospora*, *Polysporina*, *Sarcogyne* s. lat or *Sarcogyne* s. str. as monophyletic can be rejected. The harmonic mean ln-likelihoods of each topological hypothesis are represented in Tab. 1, together with the Bayes factor values resulting from the model comparisons. When testing the monophyly of *Sarcogyne*, the Bayes factor was 173.24 in favour of the best, unconstrained topology inferred by the Bayesian analysis. When testing
the monophyly of the core group of *Sarcogyne*, the Bayes factor was 328.12. And
finally, when testing the monophyly of *Polysporina* and *Acarospora*, the Bayes
factors were 166.09 and 167.78 respectively, in favour also of the best
unconstrained topology inferred by the Bayesian analysis. There is therefore very
strong evidence against all three hypotheses of monophyly (BF>>10; Kass &

Reconstruction of ancestral character states

Neither the presence of a carbonized epihymenium nor a carbonized exciple are
restricted to monophyletic groups (Fig. 3). We reconstructed the ancestral states of
these two characters in four supported nodes of the phylogeny, i.e. the node
including all Acarosporaceae except *Myriospora* (node 1, Fig. 3), the node
including *Acarospora* and *Sarcogyne* s. lat. (node 2, Fig. 3), the *Acarospora* clade,
which was only supported in the Bayesian analysis (node 3, Fig. 3), and finally the
most inclusive node within the *Acarospora* clade supported by all three
phylogenetic methods (node 4, Fig. 3). The results are summarized in Tab. 2. The
ancestral state of the carbonized exciple was reconstructed as ‘absence of
carbonized exciple’, by all methods, both for the common ancestor of the
Acarosporaceae excluding *Myriospora* at node 1, and for the common ancestor of
node 4 (i.e., the most inclusive clade within the *Acarospora* clade, supported by all
three reconstruction methods). Maximum parsimony and the two Bayesian methods
reconstruct nodes 2 and 3 as ‘absence of carbonized exciple’, although with low
probability, whereas maximum likelihood suggests a higher probability for the
common ancestor of node 2: *Acarospora* and *Sarcogyne* (s. lat.) having a non-
carbonized exciple (Table 2; Fig. 4). When studying the ancestral states of the
carbonized epihymenium we found that the common ancestor of the four nodes was reconstructed as ‘absence of carbonized epihymenium’, by all methods. BayesTraits, however, attributed very low probability to this state in the case of the ancestor of the *Acarospora* clade, including *Polysporina simplex* WE30, *P. simplex* SAR236, and *Polysporina* sp. 2 (Tab. 2; node 3, Fig. 3), where the probability was distributed between ‘presence’ and ‘absence’ of carbonized epihymenium. The total number of transformations calculated using parsimony (Mesquite) and stochastic mapping (SIMMAP) are included in Tab. 3. The most probable number of changes in the carbonized exciple is 12 according to parsimony, and 28 under the Bayesian approach, whereas the most probable number of changes in the carbonized epihymenium is 7 according to parsimony or 21 according to the Bayesian analysis. In all cases the numbers are several times higher than the minimum possible amount of change, which is the number of states minus one (if one state was plesiomorphic and the rest apomorphic) i.e. two changes in character 1 (carbonized exciple) and one single change in character 2 (carbonized epihymenium). In the evolution of the exciple, the majority of change corresponds to gains of carbonized exciple (83% under parsimony and 46% in the Bayesian method). Both methods assign some probability to other transformation types, i.e., transformations from absence of carbonized exciple to tar-like exciple; from a carbonized to a non-carbonized exciple, and transformations from carbonized exciple to tar-like exciple. Only the Bayesian method assigns some probabilities also to losses of tar-like exciple. In the second character, the gain of carbonized epihymenium accounts for all the change recovered by the parsimony method. The Bayesian method, however, distributes the probability between the two possible transformation types (gains and losses of a carbonized epihymenium) although the
highest probability (62%) is still assigned to transformations from a non-carbonized
to a carbonized ephymenium (Tab. 4).

Discussion

The taxon sampling of Acarosporaceae in this study is by far the largest included in
a phylogenetic analysis, to date. Although the sampling possibly still is uneven and
clearly biased geographically with the majority of the specimens from Scandinavia
and to a lesser extent from North America, we think that the resulting pattern
reflects the phylogeny of the group well, and that the conclusions we draw will hold
when sampling is extended further. However, some caution is necessary when
considering the current phylogeny as three of the nodes in the backbone are
supported by PPs only. It is frequently observed that Bayesian methods of
phylogenetic inference produce higher probability values (PPs) for trees or clades
than other methods such as maximum parsimony or maximum likelihood
bootstrapping. This has been attributed to the so-called “Bayesian star-tree paradox
artefact”, i.e., most implementations of Bayesian inference do not consider
polytomies during the MCMC search, and can return high PPs for branches that are
unsupported by the data (Lewis et al., 2005; Yang 2008). It is also known that
“ancient rapid radiations”, detected in phylogenies as long ingroup branches
intercalated among short backbone internodes, cause problems in phylogenetic
reconstructions (Jian et al., 2008). This effect is often associated as well to a long
phylogenetic root (Rothfels et al., 2012).

As far as it is possible to compare, our results are congruent with earlier
Myriospora is the sister-group to the rest of the family. The phylogeny furthermore
shows that species with carbonized ascomata within the Acarosporaceae are
distributed in two main clades, which are here referred to as the Acarospora and the
Sarcogyne clade respectively (Fig. 2). The Acarospora clade was retrieved in all
analyses with strong support although the inclusion of a small group of Polysporina
(P. simplex “C”) was only supported by PPs. The Sarcogyne clade lacks support for
the basal branches and there are several species, including the type of Sarcogyne,
that has an uncertain position. These results also largely agree with the recent
analysis by Miadlikowska et al (2014) who found one Acarospora s. str. clade and
one clade including both Sarcogyne and several Acarospora spp. but in addition
found a third main clade including a paraphyletic Pleopsisidium together with the A.
smaragdula group (=Myriospora). That Acarospora is paraphyletic has earlier been
indicated by Reeb et al. (2004) and Crewe et al. (2006). However, a well-supported
monophyletic group corresponding to Acarospora s. str. was found in both
investigations. Here, we suggest that the whole Acarospora clade, which includes
the type of Polysporina, in the future is treated as Acarospora. There are, however,
to our current knowledge, no phenotypic synapomorphies unique to Acarospora.
This is irrespective of if Polysporina is included in Acarospora, or not.
Characterizing the genus morphologically is not easy but may possibly be done
using a suite of characters including secondary chemistry, conidia, ascus type and
other hymenial characters. Well-known and characteristic species and species
groups belonging to Acarospora s. str. include the A. schleicheri group (the type
species and a few other terricolous taxa with globose-subglobose spores), the A.
fuscata group (brown, saxicolous species containing gyrophoric acid), yellow
species (rhizocarpic acid), e.g., A. heufleriana and some lobate species, e.g., A.
molybdina and A. wahlenbergii although it should be noted that A. molybdina was
found to belong to the Myriospora clade by Miadlikowska et al (2014).

The Sarcogyne clade as a whole has no support and is not retrieved in all
individual-gene analyses (see Suppl. Figs. 1–4). The basal branches, e.g. the
Polysporina cyclocarpa group as well as Sarcogyne clavus and S. hypophaeoides all
change position (always without support) between the four different single-marker
analyses. Despite the fact that the group has no support, we believe that additional
data are likely to indicate that this clade is real. Reeb et al. (2004) also found a very
similar Sarcogyne clade, which likewise was poorly supported basally. Their
Sarcogyne clade included Sarcogyne regularis and S. similis together with A.
cervina, A. canadensis, A. laqueata, A. macrospora and Glypholecia scabra. Our
study thus has a similar but likewise unsupported result. In our tree (Fig. 2) several
other species group in this Sarcogyne clade, but without support. An additional
number of Acarospora spp., e.g. A. glaucocarpa, A. moenium, A. insolata and A.
impressula,, and a group of Polysporina specimens including P. cyclocarpa
characterized by comparatively stout paraphyses and broad spores may thus belong
here. The Sarcogyne clade contains both cryptolecanorine and lecideine taxa and
species with or without carbonized ascomata or carbonized epihymenium. There are
a couple of general phenotypic trends characterizing this clade compared to the
Acarospora clade. All species in the Sarcogyne clade in the tree lack pigments
(other than melanins) and other secondary metabolites with the exception of
Glypholecia scabra (gyrophoric acid). All species also have a euamyloid hymenium
except for the Polysporina cyclocarpa group, which have a hemiamyloid
hymenium. However, species without pigments and with a euamyloid hymenium
are also present in the Acarospora clade. Larger and broader spores, stout
paraphyses and a low hymenium are also characters that at least appear to be more common in the *Sarcogyne* clade as compared to the *Acarospora* clade. With the current dataset, there is in any case no support for the *Sarcogyne*-clade as such, and little support for the relationships within it. Thus it is not meaningful at present to discuss groupings in the *Sarcogyne* clade but we predict that in the future this group will best be divided in several genera. It is, however, quite likely that the topology will change considerably with more data and a revised analysis will require additional markers to result in a better supported phylogeny.

Two species not included in our earlier phylogenies occur outside both the *Acarospora* and the *Sarcogyne* clades, viz *A. rhizobola* and *A. terricola*. These two terricolous taxa are both characterized by having bacilliform conidia, a character that is unusual within the Acarosporomycetidae and is, in addition to these two species, known from *A. benedarensis* (Knudsen and Fox 2010), *A. convoluta* (Magnusson 1929), *A. oligyrophorica* (Aptroot 2002), *A. sphaerosperma* (Knudsen et al. 2010), *Sarcogyne crustacea* (Knudsen and Kocourková 2010) and the two monotypic genera *Lithoglypha* and *Thelocarpella* (Brusse 1988, Navarro-Rosinés et al. 1999). A recent publication by Gueidan et al. (2014), found *A. rhizobola* to be closely related to *Thelocarpella gordensis* as well as the poorly understood *Trimmatothelopsis versipellis* which was also found to have bacilliform conidia. The relationship between these species will be investigated further in a forthcoming paper.

The surprising conclusion from this investigation is that carbonized or melanized ascomata is a highly plesiomorphic trait in the Acarosporaceae. Strongly black pigmented ascomata have clearly appeared independently numerous times in the evolution of the family. Furthermore, *Polysporina* with its typical umbonate,
carbonized apothecia is polyphyletic, and most species are not closely related to
*Sarcogyne* as previously believed. Instead, they are nested within *Acarospora* in a
strict sense. In addition, the species in *Sarcogyne* with a strongly carbonized
apothecium margin do not form a monophyletic group. These species were believed
to make up the core group of *Sarcogyne* and includes the type species *S. clavus*. The
results clearly show that neither the presence of a carbonized exciple nor the
presence of a carbonized ephymenium characterize a natural group in this lichen
family and thus is of very limited use to characterize genera or indeed the generic
placement of any formal taxa. A weak carbonization (“melanization”) of the
apothecial margin has also been noted to sometimes occur in several species in
*Acarospora* and *Myriospora* (Knudsen 2007a, Knudsen & Flakus 2009, Westberg et
al. 2010), which supports this.

The distinction between lecanorine and lecideine ascomata within
*Acarosporaceae* is usually clear-cut and has been an important character to separate
*Acarospora* (lecanorine) from *Polysporina* and *Sarcogyne* (lecideine). However,
some problematic cases have been observed. Magnusson placed a few taxa, today
recognized as *Polysporina*, in *Acarospora* based on the observed presence of green
algae in the margin (Magnusson 1924, 1929, 1935). These species otherwise have
the typical carbonized ascomata of *Polysporina*. Later authors have not commented
on this, either not observing the presence of green algae or possibly considering it
an occasional or aberrant occurrence and included them as *Polysporina*. In this
study we have found several specimens of *P. subfuscescens* s. lat., with green algae
within the carbonized margin (Fig. 1E–F). In the analysis they group with
specimens with distinctly lecideine ascomata, identified either as *P. subfuscescens*
or *P. simplex* depending on whether they were interpreted as lichenicolous or not.
The lichenine specimens are all clearly lichenicolous, developing on the thallus of a host. It appears to us that this character is the result of different developments or different stages of the infection of the host.

The position of the type species of *Polysporina*, *P. simplex* has not been clear in earlier studies. *Polysporina cf. simplex* was found to be sister species to the rest of the *Acarosporaceae/Acarosporomycetidae* by Reeb et al. (2004) but other specimens sequenced and not included in that paper did not group together (Reeb, *pers. comm.*). A different position of *P. simplex* was found by Crewe et al. (2006) but according to our observations that specimen is a misidentified *Sarcogyne cf. clavus*. In this analysis we have identified *P. simplex* according to recent morphological hypothesis (Knudsen & Kocourková 2008, 2009). Samples attributed to *Polysporina simplex* are found in three different clades in our phylogeny (Fig. 2). One clade (*P. simplex “C”*) is the sister clade to the rest of the *Acarospora* clade and the remaining two are nested inside the *Acarospora* clade. The *P. simplex* specimens differ between the clades in ecology and hymenium height (Fig. 1A–D) and we conclude from our studies of the type material that it belongs to either of the two closely related clades within *P. simplex “A”*, which are close to the generic type of *Acarospora, A. schleicheri.* (Fig. 2), *Polysporina* will be thus be treated as a synonym to *Acarospora*. It is also clear that also *P. subfuscens* in its current concept is not monophyletic and that a revision of the species taxonomy thus is necessary. Our morphological studies so far indicate that it is not a case of cryptic species but rather that the morphological characters have not been well understood. Important characters not emphasized in the current broad concepts of some species include hymenium height, spore width and paraphysis thickness. The taxonomic implications will be addressed in a separate paper.
In this paper, we have again found evidence suggesting that traits seen as extremely important in current generic and species classification of fungi including lichens, do not necessarily characterize natural, monophyletic groups, as in so many other investigations (e.g. Wedin et al. 2004; Lumbsch and Leavitt 2011; Spribille and Muggia 2013; Otálora et al. 2013, 2014; Ekman et al. 2014). Strongly black pigmented (carbonized or melanized) ascomata have independently arisen numerous times in the evolution of the Acarosporomycetidae/Acarosporaceae, and the genera *Sarcogyne* and *Polysporina* are thus distinctly non-monophyletic. Ancestral state reconstructions showed that carbonization of the hymenium was absent at all reconstructed nodes, and carbonisation of the exciple was absent at two nodes and equivocal in the other two. The number of changes in character states of both carbonized epihymenium and carbonized exciple are considerably more than the minimum number, showing that these characters are highly likely to change during the evolution in this group, again making them unlikely to characterize natural groups on higher level. Carbonization seems to be a derived character that has appeared during particular episodes in the evolution of the Acarosporomycetidae/Acarosporaceae. Melanin has been connected with protection against both metals and solar energy, because of its metal-binding capacity (McLean et al., 1998; Purvis et al., 2004) and its ability to absorb UV radiation (Gauslaa & Solhaug, 2001; Solhaug et al., 2002; Nybakken et al., 2004). If dated and geographically wider phylogenies of the group become available in the future, it would be interesting trying to connect the gain of strongly black-pigmented ascomata with events such as colonization of more exposed or metal-rich areas, where melanin-rich structures could have been of adaptive value. Finally, the presence or absence of green algae in the ascoma margin (lecanorine or lecideoid
lecideine ascomata) seems to be variable even within single species. We can predict future substantial re-arrangements of this very diverse and evolutionary interesting group of lichenized fungi.

Acknowledgements

This study was funded by grants to Martin Westberg by The Swedish Taxonomy Initiative (Svenska Artprojektet, administered by the Swedish Species Information Centre/ArtDatabanken) and was further supported by grants to Mats Wedin from the Swedish Research Council (VR 621-2009-5372, VR 621-2012-3990). The work of Kerry Knudsen was financially supported by the grant “Environmental aspects of sustainable development of society” 42900/1312/3166 from the Faculty of Environmental Sciences, Czech University of Life Sciences Prague. We are grateful to the staff at the Molecular Systematics Laboratory at the Swedish Museum of Natural History for laboratory assistance, in particular Jan Ohlson and Bodil Cronholm. Valerie Reeb kindly shared unpublished details from on her work on Acarosporaceae. The first author would finally like to thank Ulf Arup (LD), Philippe Clerc (G), Leif Tibell (UPS), Toni Berglund (Karlskoga) and members of the Swedish Lichen Society for assistance during field work.
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phylogenetic studies of the euascomycetes (Pezizomycotina Fungi) with special emphasis on the lichen–forming Acarosporaceae and evolution of polyspory.


Table 1. Comparison of three topological hypotheses; 1) *Sarcogyne* is a monophyletic group, 2) the core group of *Sarcogyne* is monophyletic, and 3) *Polysporina* is a monophyletic group, with 4) the best topological hypothesis inferred by the Bayesian analysis, when using an unconstrained model (i.e., not assuming monophyly for any of the groups). Marginal likelihoods of each model were estimated as the ln harmonic mean likelihoods of the data. Bayes factors are calculated as twice the difference of the ln harmonic mean likelihoods of the two models being compared.

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