The family Physciaceae in Fennoscandia: phylogeny inferred from ITS sequences

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Abstract: The internal transcribed spacers (ITS1 and ITS2), and the 5.8S region of the nuclear ribosomal DNA were sequenced for 52 specimens representing 35, mostly foliose, taxa of the lichen family Physciaceae (Lecanorales). The sequences were phylogenetically analyzed using parsimony jackknifing. Xanthoria parietina and X. sorediata were chosen as outgroups. Because of the variable nature of the ITS regions parsimony jackknifing analyses were performed on different alignments. The analyses resulted in trees with several shared, well-supported groups. A consensus tree, summarizing all the trees from different alignments was also calculated. In that tree the family Physciaceae formed a monophyletic group. Some of the crustose species of Physciaceae (in the genera Amandinea, Buellia and Dimelaena) appeared as two sister groups to the rest of the family. In this latter group Physcia, Heterodermia speciosa and Mobergia calculiformis constituted the sister group to the remaining family, which included the genera Anaptychia, Hyperphyscia, Phaeorrhiza, Phaeophyscia, Physconia, and Rinodina. The genus Phaeophyscia was found to be monophyletic with 100% support. Anaptychia and Physconia, together with Phaeorrhiza nimbosa and Rinodina turfacea formed its sister group. Hyperphyscia adglutinata was found in a trichotomy with the other two groups.

Key Words: Ascomycetes, Lecanorales, lichenized fungi

INTRODUCTION

The family Physciaceae (Ascomycetes, Lecanorales) has a worldwide distribution with about 30 genera. Some of them are fruticose and foliose lichens [e.g., Anaptychia Körb., Physcia (Schreb.) Michx., Phaeophyscia Moberg and Physconia Poelt], while others are crustose or placoid [e.g., Buellia De Not., Rinodina (Ach.) Gray]. The family is primarily characterised by ascus and spore structure. These characters, together with pycnoconidial characters, secondary chemistry and cortical structures, have been used to separate genera within Physciaceae. Several morphological studies in Physciaceae have been carried out (Kurokawa 1962, Poelt 1965, 1974, Moberg 1977, Scheidegger and Ruef 1988, Mayrhofer et al 1996, Matzer and Mayrhofer 1996), and additional studies have evaluated the utility of some morphological characters for taxonomical purposes (Mayrhofer 1982, Ramboldt et al 1994, Nordin 1997). According to Stenroos and DePriest (1998) SSU rDNA data suggests that Physcia aipolia (Ehrh. ex Humb.) Fürn. and Santessonia namibensis Hale & Vobis, both belonging to Physciaceae, are the sister group to a species of the genus Leprocaulon Nyl. within Lecanorales. This group, in turn, forms a sister group to Teloschistales. However, Stenroos and DePriest (1998) used only two species of Physciaceae. Previously no comprehensive phylogenetic analysis of the family Physciaceae has been carried out.

This is the first attempt to examine the phylogeny of the family Physciaceae by applying cladistic methods to DNA sequence data. We restricted our study to Fennoscandia. Our goal was to reconstruct the phylogeny of the family and to find out whether the genera within the Physciaceae are monophyletic. Our research had the secondary purpose of determining whether the putative species pairs suggested by Poelt (1970) and Moberg (1977) would form monophyletic units in the analyses. Further we wanted to find appropriate outgroups for our subsequent species pair studies (Lohtander et al unpubl) and we wanted to determine whether the placoid and crustose spe-
cies within Physciaceae would fall into one or several monophyletic groups.

MATERIALS AND METHODS

Voucher specimens.—Specimens examined are deposited at the Swedish Museum of Natural History (S) or in Uppsala (UPS). D = Denmark, F = Finland, I = Iceland, N = Norway, S = Sweden. GenBank numbers are given in parentheses.

Anaptychia ciliaris (L.) Körb.: S: Upl, Tullgarn, Lohtander 343 (S) (AF224366). Anaptychia runcinata (With.) J. R. Laundon: F: Ta, Karjalohja, Lohtander & Jalonen 325 (S) (AF224355). Anaptychia variabilis (Ach.) Norman: S: Vsm, Ängby, Nordingra, Moberg 10434 (UPS) (AF224371). Anaptychia orbitalis (Neck.) Moberg: S: Upl, Uppsala, Moberg 12046 (UPS) (AF224351). Anaptychia ciliaris (L.) Körb.: S: Upl, Tullgarn, Lohtander 343 (S) (AF224366). Anaptychia var. aestivalis (Vain.) Moberg and Physcia phaea were sequenced for the analyses. The ITS region has been successfully used in several previous studies of lichens, at the species level. Other species studied were Heterodermia speciosa, some crustose lichens belonging to Physciaceae (from the genera Amandinea, Buellia, Phaeophyscia, and Mobergia calculiformis) from Baja California, Mexico. All the other specimens have been collected from Denmark, Finland, Iceland, Norway and Sweden. Xanthoria parietina and X. sorediata were chosen as outgroup specimens.

DNA techniques.—The internal transcribed spacers ITS1 and ITS2 and the 5.8S rDNA of the nuclear ribosomal DNA were sequenced for the analyses. The ITS region has been successfully used in several previous studies of lichens, at the species and genus level (Niu and Wei 1993, Goffinet and Bayer 1997, Lohtander et al 1998a, b, Myllys et al 1999a, Thell 1998), as well as at higher taxonomic level (Berbee et al 1995, Luzoni 1997, Myllys et al 1999b), in the latter case usually in combination with the small subunit (SSU) rDNA.

DNA was extracted from both fresh collections and herbarium material using The QIAamp Tissue Kit from Qiagen. The oldest specimen from which DNA was successfully extracted was from 1980. For extraction we used small fragments of thalli, or apothecia. The procedure was performed according to the manufacturers’ instructions with the following modifications. The tissue was macerated with a mini-pestle in 50 µL of buffer AT, omitting the liquid nitrogen phase. After maceration, 130 µL of buffer AT and 20 µL Proteinase K were added, and the sample was incubated in 55–60°C overnight. The extracted DNA was eluted in 40 µL
of elution buffer AE. Before PCR the DNA was further diluted 10 times with distilled H$_2$O.

PCR-reactions were performed using Pharmacia Biotech Inc. Ready To Go PCR beads, 11.5 $\mu$L H$_2$O, 11.5 $\mu$L DNA dilution and 1 $\mu$L of 10 $\mu$M primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al 1990), in a 30-cycle reaction with a PCR profile of 60 s at 95 C (denaturation), 60 s at 60 C (annealing), and 60 s at 72 C (extension). All PCR products were purified with the QiAquick PCR Purification Kit of Qiagen, and eluted with 40 $\mu$L dH$_2$O.

Both strands were sequenced. Fluorescent labelled (Cy5) primers used for sequencing were ITS5 (White et al 1990), ITS2-KL (Lohtander et al 1998a, b) and ITS1-LM (Myllys et al 1999a). The sequencing reactions were produced using Amersham’s ThermoSequenase Sequencing Kit, in a reaction with a profile of 2 min at 95 C (1 cycle), followed by 30 s at 95 C, 30 s at 50 C, and 1 min at 72 C (30 cycles). The samples were run on a 6% Long Ranger gel in a Pharmacia Biotech Inc. ALFExpress automatic sequencer.

Sequences were aligned using the ClustalW (Thompson et al 1994) alignment program. Since different alignment parameter settings may produce different alignments and affect the tree topology, we chose an approach described by Farris et al (1999) also used by Myllys et al (1999b). Several alternative alignments were analyzed with parsimony jackknifing in order to identify groups that are shared in every tree. The following combinations were performed (gap opening penalty/gap extension penalty): 1/1, 2/4, 1/5, 3/5, 6/3, 5/5, 8/6, 5/7, 7/5, 10/1, 10/5, 9/7, 5/10, 13/5, 15/5, 10/8, 10/10, 20/10, 25/10, and 30/10. Each alignment was estimated by eye and those that appeared as clearly unrealistic (the alignment program failed to initiate the alignment from the same site at the beginning of the ITS1 region) were omitted in the analyses. A consensus of all the resulting trees was constituted, as suggested by Farris et al (1999). Sequence alignment with gap opening penalty 9.0 and gap extension penalty 7.0 is deposited in TreeBASE (S478, M700).

Phylogenetic analysis.—The data were analyzed by parsimony jackknifing (Farris et al 1996) using the computer program Xac (designed by J. S. Farris and discussed in Källersjö et al 1998), with the following settings: 1000 replicates were performed with branch-swapping and 5 random addition sequences each. In parsimony jackknifing (Farris et al 1996) the original data set is resampled so that approx 63% of the characters are chosen randomly and without replacement for parsimony analysis. Trees are calculated for each replicate and combined into jackknife trees showing only groups that are present in at least 50% of the replicates. One of the benefits of using Xac is its speed compared to other programs. Furthermore, uninformative characters do not affect support values in parsimony jackknifing as they do in bootstrapping (Carpenter 1996). Gaps were treated as a fifth character state or coded as missing data.

RESULTS

The ITS sequences obtained were approx 500 bp, with some exceptions. *Heteroderma speciosa* had a slightly shorter ITS region (477 bp) compared to the other specimens. The sequences of *Anaptychia ciliaris* varied 532–563 bp. The aligned data matrix ranged from 584 (alignment with gap opening penalty 10; gap extension penalty 10) to 679 sites (alignment with gap opening penalty 1.0; gap extension penalty 1.0). The number of informative characters varied from 274 to 302 when gaps were treated as character states, being approx 45% of all characters. The number of gaps in alignments varied from 33 (alignment with gap opening penalty 15.0; gap extension penalty 5.0) to 79 (alignment with gap opening penalty 1.0; gap extension penalty 1.0).

Gap opening penalties above 15.0 resulted in clearly unrealistic alignments and such alignments were abandoned. Seventeen alignments were left to be used in the analyses. In addition to the consensus tree we also present the tree that had the best resolution (the number of nodes in the tree) as well as the best support (the number of nodes with support of 95% or more in the tree). The alignment with gap opening penalty 9.0 and gap extension penalty 7.0 resulted in the best resolved tree (with 37 groups) and received the best support values (16 nodes with a support of 95% or more) in the Xac analysis (Fig. 1). The consensus tree is presented in Fig. 2.

The influence of different alignment parameter settings in the trees was rather low. Conflict between the trees based on the different alignments was seen only in the *Buellia* group (Figs. 1, 2). Otherwise the topologies of all 17 trees were rather uniform. The main difference was in the degree of resolution. In all trees the family Physciaceae formed a monophyletic group with 100% support (Figs. 1, 2).

The influence of the gaps on the phylogenetic reconstruction was not drastic either. Alignment with gap opening penalty 9.0 and gap extension penalty 7.0 gave an almost identical tree regardless of whether or not the gaps where used as character states. The resolution was somewhat lower (29 groups) when the information provided by gaps was omitted. The other sixteen trees based on different alignments had more deviation in degree of resolution between the trees where gaps were treated as character states and the trees where gaps were coded as missing data. No conflict was to be observed between these trees, but the latter ones (with gaps as missing data) were much more poorly resolved compared to the trees with gaps as character states.

*Buellia* group. At the base of all trees two *Buellia alboatra* specimens formed a sister pair with 100% support (Figs. 1, 2). When the parameter settings in the alignments were low (gap opening and gap extension penalties under 3.0), *B. alboatra* formed a sister group to the rest of the family in the analysis.
Fig. 1. Jackknife tree based on ITS sequence data (alignment with gap opening penalty 9.0 and gap extension penalty 7.0). The numbers given at each node indicate the jackknife support.
Fig. 2. A strict consensus tree of seventeen jackknife trees based on different alignments.
Otherwise B. alboatra grouped with a clade containing Amandinea punctata, B. disciformis and Dimelaena oreina. Together they formed a sister group to the rest of the family with 83–99% support in all trees. The resolution within the Amandinea-Buellia-Dimelaena-clade varied from one tree to another.

Physcia group. The next clade (Figs. 1, 2) contained the genus Physcia, and the species Heterodermia speciosa and Mobergia calcifer (the Physcia group) forming the sister group to Phaeophyscia (the Phaeophyscia group) and the genera Anaptychia, Hyperphyscia, Phaeorrhiza, Physconia and Rinodina (the Physconia group).

The genus Physcia either formed a monophyletic group together with Heterodermia speciosa and Mobergia calcifer (Fig. 1) or formed a polytomy with those two species and the rest of the family (Fig. 2). The support for a monophyletic Physcia was always above 90%. In the best tree (Fig. 1) Physcia magnussonii appeared as a sister taxon to the rest of Physcia. There were two large clades within Physcia in most of the trees. The first clade, which was present in all trees (with 95–100% support) contained all the specimens of Physcia aipolia and Physcia caesia in two distinct groups. One with Physcia aipolia var. alnophila had a 100% support. The other with Physcia aipolia var. aipolia grouping together with two unresolved Physcia caesia specimens had a 91–96% support in all trees (Figs. 1, 2).

The second clade contained the ciliate species Physcia adscendens, Physcia leptalea, Physcia tenella var. tenella and Physcia tenella var. marina. Physcia leptalea formed a monophyletic group (Figs. 1, 2), as did Physcia adscendens (Fig. 1) and Physcia tenella (trees not shown) in some cases. The clade containing Physcia adscendens, Physcia leptalea and Physcia tenella collapsed when gaps were coded as missing data (trees not shown).

Physcia dimidiata var. ornata, Physcia dubia, and Physcia stellaris remained unresolved in many cases (Figs. 1, 2), as did Physcia magnussonii (Fig. 2), but in some of the trees they appeared as unresolved taxa in a clade containing the group with Physcia adscendens, Physcia leptalea and Physcia tenella (trees not shown).

Phaeophyscia group. The species of Phaeophyscia formed a monophyletic group with 100% support in all trees (Figs. 1, 2). Phaeophyscia nigricans appeared as a sister taxon to the rest of the genus. Phaeophyscia constipata, Pha. endophoenica and Pha. endococcina either formed a monophyletic group (Fig. 1) or were in a polytomy with the group containing the rest of Phaeophyscia (Fig. 2). In the best tree Pha. constipata and Pha. endophoenica grouped together with two Pha. endococcina specimens (Fig. 1). Phaeophyscia distorta, Pha. orbicularis and Pha. sciastra formed a monophyletic group in every analysis (Figs. 1, 2). Two Pha. sciastra specimens appeared as a sister group to the other two species in some alignments (trees not shown) or they formed a trichotomy at the base of the next group (Figs. 1, 2). Phaeophyscia ciliata and Pha. orbicularis formed a well supported monophyletic group in each tree. The two Pha. orbicularis specimens formed a monophyletic group within that group with 90–91% support in all trees.

Physconia group. The sister group to the Phaeophyscia group contained the genera Anaptychia, Phaeorrhiza, Physconia and Rinodina (the Physconia group; Figs. 1, 2). Hyperphyscia adglutinata was either found as a basal sister taxon in the Physconia group (Fig. 1) or in trichotomy with the two groups (Fig. 2), depending on the alignment used in the analysis. Rinodina turfacea and Phaeorrhiza nimbosa formed a grade basal to the genera Anaptychia and Physconia. Anaptychia ciliaris either formed a sister group to the rest of the specimens (Fig. 1) or remained unresolved with A. runcinata and Physconia grisea (Fig. 2). Anaptychia ciliaris always formed a monophyletic group with 100% support. In the best tree Anaptychia runcinata, Physconia grisea and Physconia perdisiosa formed a grade basal to the group containing the rest of the Physconia specimens (Fig. 1). Physconia muscigena and Physconia enteroxantha were in polytomy with a group containing Physconia distorta and Physconia detersa as a sister group to another Physconia distorta specimen.

DISCUSSION

Various approaches have been employed to analyze sequence alignments for phylogenetic purposes. Alignments are either used in analyses as they are, or they may be manipulated in various ways. For example, in order to avoid noisy characters, Berbee et al (1995) and Luzoni (1997) adjusted their alignments, pruning off regions with long gaps, or regions otherwise considered ambiguous. The problem with such an approach is that a part of the information is lost, since such regions may contain phylogenetic structure in addition to noisy characters (Myllys et al 1999b). Furthermore, noise in a data set may not be a severe problem, since Wenzel and Siddall (1999) have shown that a large fraction of noisy characters are needed to produce loss of phylogenetic signal. We left our alignments intact, since we wanted to use all available data.

Gaps are often coded as missing data. It is a simple way to eliminate artifacts based on ambiguous alignments, but the disadvantage in such an approach is that information may be lost. Another method is to
treat gaps as a fifth character state. The information provided by indels remains intact, but then long gaps may seem to present a problem. Should one code a long gap consisting of several gaps (nucleotide losses) as one character (presence/absence), or should each single position in a gap equal one character? The difficulty in the first alternative is how to treat overlapping gaps. In the second alternative the gaps may gain too great significance in the analysis. Since our alignment contained many overlapping gaps of which homology was difficult to assess, we coded each single gap as a character state. The different alignments yielded trees that were not in conflict with each other whether or not gaps were coded as fifth character state, but the trees where gaps were coded as fifth character state were better resolved than the other trees. The better resolution seemed not to be an artifact only, since different alignments yielded similar groups in each analysis. This indicates that the ambiguous parts contained phylogenetic signal. Furthermore, the well-supported group containing Physcia adscendens, Physcia leptalea and Physcia tenella (Figs. 1, 2), species with shared morphological character (cilia), collapsed when gaps were coded as missing data.

The alignment parameter settings had some, although not a drastic influence on the resulting trees. The Buellia group was affected by different alignment parameter settings. Perhaps more comprehensive sampling would make this group more stable. Otherwise the overall structure in each tree remained similar. Myllys et al (1999b) also observed in their Arthoniales study that the trees based on different alignments had similar topologies and were not in conflict, despite their highly variable ITS sequence data (up to 85% of the characters were informative, compared to 45% in our present data set). Only the degree of resolution in the trees was variable.

Growth form has traditionally been one of the grounds for grouping lichenized fungi. According to our study crustose (or foliose) growth forms has arisen at least twice within Physciaceae (Figs. 1, 2). The species in the Buellia group are crustose, as are the basal taxa (Rinodina and Phaeorrhiza) in the Physcionia group. Stenroos and DePriest (1998) and Myllys et al (1999b) have also observed multiple origins of different growth forms in their studies. Our result together with the results of Stenroos and DePriest (1998) and Myllys et al (1999b) suggest that the growth form of lichens may not be a useful character for phylogenetic studies at higher taxonomic levels, either in ascohymenial (Lecanorales) or ascolocular (Arthoniales) lichens.

Ramboldt et al (1994) have studied the ascus types of several species in Physciaceae and found that two kinds of asci (with some intermediate types) are present in the family: Baciodia-type asci and Lecanora-type asci. In our analysis the Buellia group corresponds to the Baciodia-type of asci, while the rest of the species have asci of the Lecanora-type. Furthermore Ramboldt et al (1994) suggested that the genus Dimelaena is more closely related to Buellia than to Rinodina. Our result is in agreement with that. The taxon delimitation in Physciaceae is still somewhat unclear. For example, the genus Buellia appeared not to be monophyletic. Also the relationship of Anaptychia and Physcionia remained unresolved. Anaptychia did not form a monophyletic group in any of the analyses and Physcionia formed a monophyletic group in some cases only (Fig. 1).

The species pair concept was originally described by Poelt (1970). It is a well known concept in lichenology based on the observation that morphologically indistinguishable taxa may be grouped as pairs—one member reproducing sexually and the other asexually. Our results suggest that the putative species pair Physcia distorta (sexual) and Physcia detersa (asexual, Moberg 1977) might represent a single species, but more sampling is needed to confirm this. Our result also indicates that Physcia aipolia (sexual) and Physcia caesia (asexual) should be treated as a single species since Physcia caesia is nested within Physcia aipolia and varietal placement is not consistent (Figs. 1, 2). Physcia tenella (asexual) and Physcia leptalea (sexual), as well as Physcia dubia (asexual) and Physcia stellaris (sexual), are supposed to be closely related to each other and forming species pairs (Moberg 1977) but the ITS data set failed to either confirm or contradict that (Figs. 1, 2). The genus Physcia and some species pairs within it are being studied separately (Lohtander et al unpubl).

Phaeophyscia appeared as a well-defined group, having 100% support regardless of the alignment used. Until this genus was described by Moberg (1977) the species of Phaeophyscia were included in the genus Physcia (Poelt 1974). According to our results Phaeophyscia is more closely related to the Physcionia group than it is to Physcia. The putative species pair Phaeophyscia ciliata (sexual)—Pha. orbicularis (asexual, Moberg 1977) consistently formed a monophyletic group. These species will be studied separately (Lohtander et al unpubl). In Phaeophyscia some of the specimens representing the same species, such as Pha. endococcina, Pha. ciliata and Pha. sciastra, did not always group together (Figs. 1, 2). This may be due to the low number of informative characters in this group, but it is also possible that the species delimitations require further study.

The overall structure of the trees (Figs. 1, 2) cor-
respond quite well to previous genus concepts in the family (Poelt 1965, 1974, Moberg 1977). Many infrageneric relationships remain to be resolved, especially those in the genus Physcia. However, the ITS region contained enough information to resolve many of the other relationships, such as those between the different genera in Physciaceae. The structure of the trees appeared to be stable despite the variation in ITS sequences. We will continue our study by adding more data to the ITS data set.

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LITERATURE CITED


