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New entities in *Physcia aipolia–P. caesia* group (Physciaceae, Ascomycetes): an analysis based on mtSSU, ITS, group I intron and betatubulin sequences

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We have revisited the phylogenetic evaluation of the *Physcia aipolia–P. caesia* lichen group (sect. *Caesiae*; Physciaceae, Lecanorales) in order to investigate whether new sequence data and extensive sampling can help us to understand the phylogenetic relationships in that group. We combined partial mtSSU DNA data with two previously used nuclear gene regions (betatubulin, ITS) and a group I intron. We also compared the resulting phylogenies with chemical and morphological characters. Altogether 52 specimens of the *P. aipolia–P. caesia* group were analysed. Direct optimization of the molecular data revealed several well-supported groups. Our results essentially agreed with those of the earlier studies, and we were able to confirm the independent taxonomic status of some controversial morphotaxa. We also discovered at least two distinct clades that potentially represent species new to science. A new nomenclatural combination, *Physcia alnophila* (Vain.) Loht., Moberg, Myllys & Tehler, is proposed.

Key words: betatubulin, intron, ITS, lichen, mtSSU rDNA, phylogeny, *Physcia*

Introduction

The lichen family Physciaceae (Ascomycetes, Lecanorales) has a worldwide distribution. The genus *Physcia*, one of the approximately 30 genera in Physciaceae, contains about 50 species worldwide (Moberg 1994). *Physcia* is separated from the other genera in the family by the *Physcia*-type ascospores, subcylindrical pycnoconidia and the presence of atranorin in the upper cortex (Moberg 1977).

The genus *Physcia* has been divided into sections (e.g., sect. *Physcia*, *Caesiae*, *Fusisporae,* and *Stellares*) by Moberg (1977), based on

morphological and chemical characters, such as the presence or absence of marginal cilia and K+ yellow colour reaction in the medulla. Our goal in the present study was to investigate the section *Caesiae*, which is characterized by maculate surface of the thallus, lobes without cilia, K+ yellow reaction in medulla, and the presence of zeorin in the thallus (Moberg 1977). The section *Caesiae* contains the widespread species *P. aipolia*, *P. caesia* and *P. phaea* (Moberg 1977). Also several other *Physcia* species, such as *P. alba*, *P. albata*, *P. alboplumbea*, *P. cinerea*, *P. convexa*, *P. coronifera*, *P. decorticata*, *P. dilatata*, *P. lanciatula*, *P. lopezii*, *P. nubila*, *P. pachyphylla*, *P. poncinsii*, *P. rolfii*, *P. sinuosa*, *P. tribacoides*, *P. undulata*, and *P. verrucosa* have a K+ yellow medulla reaction and they contain zeorin. Hence they have been included in the section *Caesiae* as well (Moberg 1986, 1989).

The ITS phylogeny by Helms *et al*. (2003) indicated that at least *P. alba*, *P. phaea* and *P. undulata* do not belong in the *P. aipolia–P. caesia* group. Furthermore, our ongoing study of the phylogeny of the genus *Physcia* indicates that at least *P. convexa*, *P. lancinulata*, *P. nubila, P. poncinsii*, *P. rolfii*, *P. sinuosa*, and *P. tribacoides* do not form a monophyletic group with *P. aipolia* and *P. caesia* (authors' unpubl. data).

Physcia is commonly cited to contain so-called species pairs. The concept of species pairs (Poelt 1970, 1972) is based on the suggestion that lichen taxa that differ from each other only or primarily by their dispersal methods may be grouped as pairs. The so-called "primary species" produces fruiting bodies and sexual spores, while its counterpart, the "secondary species", is vegetatively dispersed, usually by means of soredia (Poelt 1972). The different counterparts of species pairs have traditionally been treated and named as separate species. The study by Lohtander *et al*. (2000) showed that based on the ITS and group I intron sequences *P. aipolia* and *P. caesia* did not form a separate group in the phylogenetic tree. Moreover, Myllys *et al*. (2001) included an additional gene region (betatubulin gene) to the ITS and intron data set. The phylogenetic tree in that study had a well-resolved and supported internal structure, and the specimens of *P. aipolia* and *P. caesia* formed several, intermixed but well-supported groups.

Physcia aipolia and *P. caesia* have different morphotypes that are sometimes treated as species or varieties. For example in *P. aipolia* Moberg (1977) recognised two varieties: var. *aipolia* and var. *alnophila*. The varieties are morphologically rather distinctive. *Physcia aipolia* var. *alnophila* forms smaller, narrow-lobed thalli and smaller spores than var. *aipolia*, as well as smaller, crowded apothecia. *Physcia aipolia* var. *alnophila* also usually grows on trees such as *Alnus incana*, *Salix* spp. and *Sorbus aucuparia*, while var. *aipolia* seems to prefer *Populus tremula* (Moberg 2002). Furthermore, *P. aipolia* var. *alnophila* has a more northern distribution than var. *aipolia*. The studies by Lohtander *et al*. (2000) and Myllys *et al*. (2001) showed that var. *alnophila* and var. *aipolia* form separate groups in phylogenetic trees and are thus obviously distinct species, but the nomenclatural combination "*P. alnophila*" was not validly published.

Similarly, *Physcia caesia* has a morphotype, *P. subalbinea* (*P. wainioi*, *P. caesioalba*) that many authors have recognised, but that Moberg (1977) treated as a synonym of *P. caesia*. Typical *P. caesia* has capitate laminal soralia while *P. subalbinea* has soralia located primarily at the lobe-tips (Moberg 1977).

We wanted to find out whether the phylogeny of the *Physcia aipolia–P. caesia* group in the studies by Lohtander *et al*. (2000) and Myllys *et al*. (2001) could be further clarified using an additional gene region (mtSSU), the secondary chemistry of the specimens and more comprehensive sampling.

We used DNA data and phylogenetic methods to (1) study the status of certain species assigned to the section *Caesiae*, (2) study the species pairs and try to clarify the species boundaries in the section, and (3) find out whether chemical characters and additional molecular data can discriminate between the morphotypes in *P. aipolia* and *P. caesia*.

While studying the morphology of the specimens, we found that certain *P. aipolia* thalli had slight morphological differences as compared with the rest of the material representing the same species. For example, most of the North American specimens have apothecia that are located only in the centre of the thallus, while the rest had at least some apothecia close to the

thallus margins. Furthermore, some Russian *P. aipolia* var. *alnophila* specimens had conspicuously minute thalli compared to the rest of the var. *alnophila* material. We especially wanted to find out whether the deviating specimens form distinct phylogenetic groups and thus perhaps represent unrecognised species.

In order to scrutinize these issues, we added several new specimens to the material of our previous studies and sequenced a partial mitochondrial small subunit (mtSSU) for most of the specimens. Partial betatubulin sequences of many of the specimens were already available in the GenBank (*see* Myllys *et al*. 2001). Similarly, internal transcribed spacers (ITS) of the nuclear ribosomal DNA, as well as a nucleotide insertion identified as a group I intron at position 1516 in SSU rDNA for many of the specimens had already been sequenced (Lohtander *et al*. 2000, Myllys *et al*. 2001).

Methods

Several new ITS, intron and betatubulin sequences were prepared for the present study and the secondary chemistry of all available specimens was studied using thin-layer chromatography.

TLC

The secondary chemistry of the *Physcia* specimens was determined by thin-layer chromatography (TLC) using standard methods (solvents A and B; *see* Culberson 1972).

Molecular techniques

The total DNA of lichen samples was extracted using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions with slight modifications described in Lohtander *et al*. (2000).

mtSSU

The small subunit of the mitochondrial ribosomal repeat (mtSSU) was amplified using the primers mtSSU1-KL and mtSSU2-KL (Lohtander *et al*. 2002). The products were sequenced using the same primers and two additional primers (mtSSU2 and mtSSU2r; *see* Zoller *et al*. 1999).

Betatubulin

Betatubulin primers for PCR and sequencing were Bt3-LM and Bt10-LM (for further information, *see* Myllys *et al*. 2001).

ITS and intron

The internal transcribed spacers ITS1 and ITS2 and the 5.8S rDNA of the nuclear ribosomal DNA, as well as insertions at position 1516 in the SSU rDNA region were sequenced using the primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). In cases where products of different lengths appeared after PCR, a rerun of samples was performed using primers ITS1-LM (Myllys *et al*. 1999) and ITS4. By using the primer ITS1-LM we were able to omit introns at the end of the SSU rDNA region and thus get a single product. All the resulting PCR products (consisting of ITS and intron) were sequenced using primers ITS5 (White *et al*. 1990) and ITS2-KL (Lohtander *et al*. 1998). However, most of the ITS sequences used in this study were obtained from our previous studies of the family Physciaceae (Lohtander *et al*. 2000, Myllys *et al*. 2001) (*see* Table 1 for the GenBank numbers).

All PCR reactions were performed using Amersham Pharmacia Biotech Inc. Ready To Go PCR beads following a procedure described in Lohtander *et al*. (2000). The PCR profile for each reaction was 60 sec at 95° (denaturation), 60 sec at 58° (annealing), and 60 sec at 72° (extension), 30 cycles, followed by 7 min at 72°. PCR products were purified with the PCR Purification Kit (Qiagen). The amplified DNA products were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequence reactions were then purified using the AutoSeq™ G-50 columns of Amersham Pharmacia Biotech Inc. The purified samples were then run on an ABI Prism 377 automated sequencer (PE Biosystems).

Table 1. Voucher specimens. The specimens examined are deposited in the Finnish Museum of Natural History, Helsinki (H), Swedish Museum of Natural History (S), Museum of Evolution, Uppsala (UPS), or Saint Petersburg University Herbarium (LECB).

No.	species	Collector	Collection no.	Herb.	mtSSU	Bt	ITS	Intron
1	*Phaeophyscia ciliata*	Himelbrant	K-04-23	LECB	EU682114	EU682154	EU682182	–
2	*Heterodermia erinacea*	Moberg	12212	UPS	EF582789	EU682155	EF582746	–
3	*Anaptychia isidiza*	Himelbrant	K-04-10	LECB	EF582828	EU682156	EF582780	–
4	*Physcia stellaris*	Moberg	12012	UPS	EU682115	EU682157	EU682183	–
5	*Physcia adscendens*	Moberg	12260	UPS	EU682116	EU682158	EU682184	–
6	*Physcia aipolia*	Hernandez&Sicilia	XII.2002	UPS	EU682117	EU682159	EU682185	–
7	*Physcia subalbinea*	Pykälä	9712	H	EU682118	EU682160	EU682186	–
8	*Physcia subalbinea*	Moberg	12019	UPS	–	AF264090	AF224384	AF224384
9	*Physcia subalbinea*	Lohtander	316	S	EU682119	AF264089	AF224385	AF224385
10	*Physcia subalbinea*	Haikonen	21103	H	EU682120	EU682161	EU682187	–
11	*Physcia subalbinea*	Vitikainen	14417	H	EU682121	EU682162	EU682188	–
12	*Physcia subalbinea*	Haikonen	12522	H	EU682122	EU682163	EU682189	–
13	*Physcia aipolia*	Tehler	7894b	S	EU682123	AF264099	AF224395	AF224395
14	*Physcia aipolia*	Tehler	7906	S	–	AF264097	AF224397	AF224397
15	*Physcia aipolia*	Tehler	7891	S	EU682124	AF264096	AF224396	AF224396
16	*Physcia aipolia*	Lohtander	404	S	–	AF264103	AF224392	AF224392
17	*Physcia aipolia*	Lohtander	314	S	–	AF264102	AF224390	AF224390
18	*Physcia aipolia*	Lohtander	400	S	–	AF264100	AF224394	AF224394
19	*Physcia aipolia*	Hermansson	3021a	UPS	EU682125	AF264101	AF224443	AF224443
20	*Physcia aipolia*	Urbanavichine	C-01333	LECB	EU682126	EU682164	EU682190	–
21	*Physcia aipolia*	Moberg	12008	UPS	EU682127	–	AF224391	AF224391
22	*Physcia caesia*	Nordin	3847	UPS	EU682128	AF264092	AF224438	AF224438
23	*Physcia* sp. 1	Moberg	10137	UPS	EU682129	AF264095	AF224431	AF224431
24	*Physcia* sp. 1	Tehler	7915	S	–	AF264094	AF224393	AF224393
25	*Physcia* sp. 1	Nordin	5160	UPS	EU682130	AF264093	AF224432	AF224432
26	*Physcia* sp. 1	Nordin	5251	UPS	EU682131	EU682165	EU682191	–
27	*Physcia* sp. 1	Nordin	5183	UPS	EU682132	EU682166	EU682192	–
28	*Physcia caesia*	Mattson	3341b	UPS	EU682133	AF264104	AF224436	AF224436
29	*Physcia caesia*	Hansen	ex.782	UPS	EU682134	EU682167	EU682193	–
30	*Physcia caesia* (non-sorediate)	Otnyukova	21	UPS	–	EU682168	EU682194	–
31	*Physcia caesia* (non-sorediate)	Otnyukova	7/24/97	UPS	–	EU682169	EU682195	–
32	*Physcia caesia*	Moberg	10810	UPS	–	AF264109	AF224436	AF224436
33	*Physcia caesia*	Tehler	7887	S	–	–	AF224387	–
34	*Physcia caesia*	Hermansson	3472a	UPS	EU682135	AF264107	AF224437	–
35	*Physcia caesia*	Lohtander	346	S	EU682136	–	AF224389	–
36	*Physcia caesia*	Myllys	190	S	EU682137	AF264105	AF224379	AF224379
37	*Physcia caesia*	Zhurbenko	96305	LECB	EU682138	EU682170	EU682196	–

38	*Physcia caesia*	Urbanavichus	C-01566	LECB	EU682139	EU682171	EU682197	–
39	*Physcia caesia*	Tehler	7886	S	EU682140	–	AF224388	AF224388
40	*Physcia phaea*	Moberg	11572	UPS	EU682141	EU682172	EU682198	–
41	*Physcia phaea*	Haikonen	17747	H	–	EU682173	EU682199	–
42	*Physcia* sp. 2	Ahti	61491	H	EU682142	EU682174	EU682200	–
43	*Physcia* sp. 2	Urbanavichus	B-021	H	EU682143	EU682175	EU682201	–
44	*Physcia alnophila*	Ahti	61479b	H	EU682144	EU682176	EU682202	–
45	*Physcia alnophila*	Hermansson	4286b	H	–	AF264087	AF224434	AF224434
46	*Physcia alnophila*	Kuznetsova	K-401	LECB	EU682145	EU682177	EU682203	–
47	*Physcia alnophila*	Urbanavichus	B-04535	H	EU682146	EU682178	EU682204	–
48	*Physcia alnophila*	Tehler	7894a	S	EU682147	AF264088	AF224383	AF224383
49	*Physcia alnophila*	Vitikainen	13191	H	EU682148	EU682179	EU682205	–
50	*Physcia alnophila*	Vitikainen	13234	H	EU682149	EU682180	EU682206	–
51	*Physcia alnophila*	Nordin	5702	UPS	–	EU682266	EU682207	–
52	*Physcia alnophila*	Kuznetsova	K-400	LECB	–	EU682181	EU682208	–
53	*Physcia alnophila*	Lohtander	399	S	EU682150	AF264085	AF224381	AF224381
54	*Physcia alnophila*	Lohtander	380	S	–	AF264086	AF224382	AF224382
55	*Physcia alnophila*	Lohtander	390	S	EU682151	AF264084	AF224380	AF224380
56	*Physcia alnophila*	Vitikainen	16135	H	EU682152	–	EU682209	–
57	*Physcia alnophila*	Ahti	64008	H	EU682153	–	EU682210	–

Alignment and phylogenetic analysis

Initially, several phylogenetic analyses of aligned data sets of the separate gene regions were performed in order to study tree topologies based on different data sets and to study the effect of indels (insertions and deletions). The sequences were aligned using the program ClustalW (Thompson *et al*. 1994). Phylogenetic trees were obtained using the heuristic search option in PAUP 4.0 (Swofford 2000), with random addition sequence and TBR. Support for each node was estimated using bootstrapping (5000 reps.), as implemented in PAUP (results not shown).

For analysing the combined DNA data set we used optimization alignment (Wheeler 1996) as implemented in POY (Gladstein & Wheeler 1997). The basic principle of optimization alignment is that alignment and search for the optimal topology are made simultaneously. This is to find topology(-ies) and alignment(s) that minimize transformations of the terminals. POY analyses were performed using the IBMSC parallel supercomputer, located at CSC — Scientific Computing Ltd., Espoo, Finland. Insertions and deletions were counted equally as transformations between sequences. *Phaeophyscia ciliata*, *Heterodermia erinacea*, *Anaptychia isidiosa*, as well as *Physcia adscendens* and *P. stellaris* were chosen as outgroups based on earlier studies of the family (e.g., Lohtander *et al*. 2000, Grube *et al*. 2001, Helms *et al*. 2003).

Results

Secondary chemistry

Physcia stellaris and *P. adscendens* contained atranorin only, while all the ingroup specimens contained atranorin and zeorin. There were also several unknown substances present in some of the specimens (*see* below).

mtSSU

The sequenced mtSSU rDNA region was 1095–1112 bp long. Most of the *P. aipolia* var. *aipolia* and *P. subalbinea* specimens had a 14–15 bp

deletion in the mtSSU rDNA region as compared with the other sequences (including the outgroup).

Betatubulin

The Bt sequences were 711–718 bp long, and they contained two introns (*see* also the study of Myllys *et al*. 2001) that were included in the analyses. Some *Physcia aipolia* var. *alnophila* specimens (11–16) contained a deletion of 7 bp in the second intron region compared to the other sequences.

ITS

The ITS region of the specimens was 478–483 bp long, but the length differences were due to few short insertions/deletions. Therefore the ClustalW alignment was rather unambiquous.

Intron

Most of the *Physcia* specimens possessed insertions in their nuclear SSU rDNA at position 1516. The length of the insertions varied from 223 to 237 nucleotides. However, the intron sequence is not available for all specimens, since some of the specimens appeared to have two types of the SSU rDNA region, which resulted in two DNA-bands of different length in PCR amplification. In such cases we used PCR primers that omit the intron region altogether, and only the ITS region was amplified and sequenced.

When visually inspected, there was a significant incongruence between the PAUP trees based on nuclear and mitochondrial data. However, the separate analyses each produced a tree that had the same terminal groups than those in the combined analysis (Fig. 1). The main discrepancy between the analyses was the order of the deep nodes. In all trees based on nuclear data sets (betatubulin, ITS, intron) *P. aipolia* var. *alnophila* formed a sister group to the rest of the specimens in the section *Caesiae*, while in the mitochondrial tree *P. subalbinea* and *P. aipolia* formed a sister group to the rest of the specimens.

Separate and combined phylogenies

We performed two kinds of POY analyses. The first data set (Fig. 1) contained all the sequenced gene regions. POY analysis with 58 taxa resulted in 12 equally parsimonious optimizations (trees) of 2019 steps (CI 48; RI 96).

Since the use of introns may give misleading information (Simon *et al*. 2005) we also performed a second analysis, in which the intron data set was excluded. That tree had a slightly poorer resolution and support.

The ingroup (sect. *Caesiae*) of the phylogenetic tree (Fig. 1) consists of six main groups, which are discussed below.

Group I: *Physcia subalbinea*

The group had a Bremer support value of 6, and the sorediate specimens form a subgroup that has a support value of 21. All specimens in this group have soralia that are located at lobe tips (hence typical *P. subalbinea*), except for specimen 6, which contains apothecia. The specimens were collected in Finland, Macaronesia (apothecial specimen 6), Russia and Sweden. The specimens were growing on *Chamaecytisus* (specimen 6) or rocks (mostly granite). This group is especially rich in secondary substances. All sorediate specimens in the group contain atranorin and zeorin (the apothecial specimen 6 was not available for TLC). There are also additional substances. Specimens 7, 9, 10 and 11 contain the substance "X-unknown" that is slightly fluorescent (blue) and with Rf class values of 2 (solvent A) and 1.5 (solvent B). Many of the specimens also contain an unknown substance that is only observable in the solvent B (Rf 5) or two unknown UV+ substances that have Rf values of 4 (solvent A) and 5.5 (solvent B); 6.5 (solvent A) and 6.5 (solvent B).

Group II: *Physcia aipolia*

Group II contains specimens that are morphologically closest to *P. aipolia s. stricto*, having numerous apothecia of variable size. The specimens are from Finland, Sweden and Russia, and

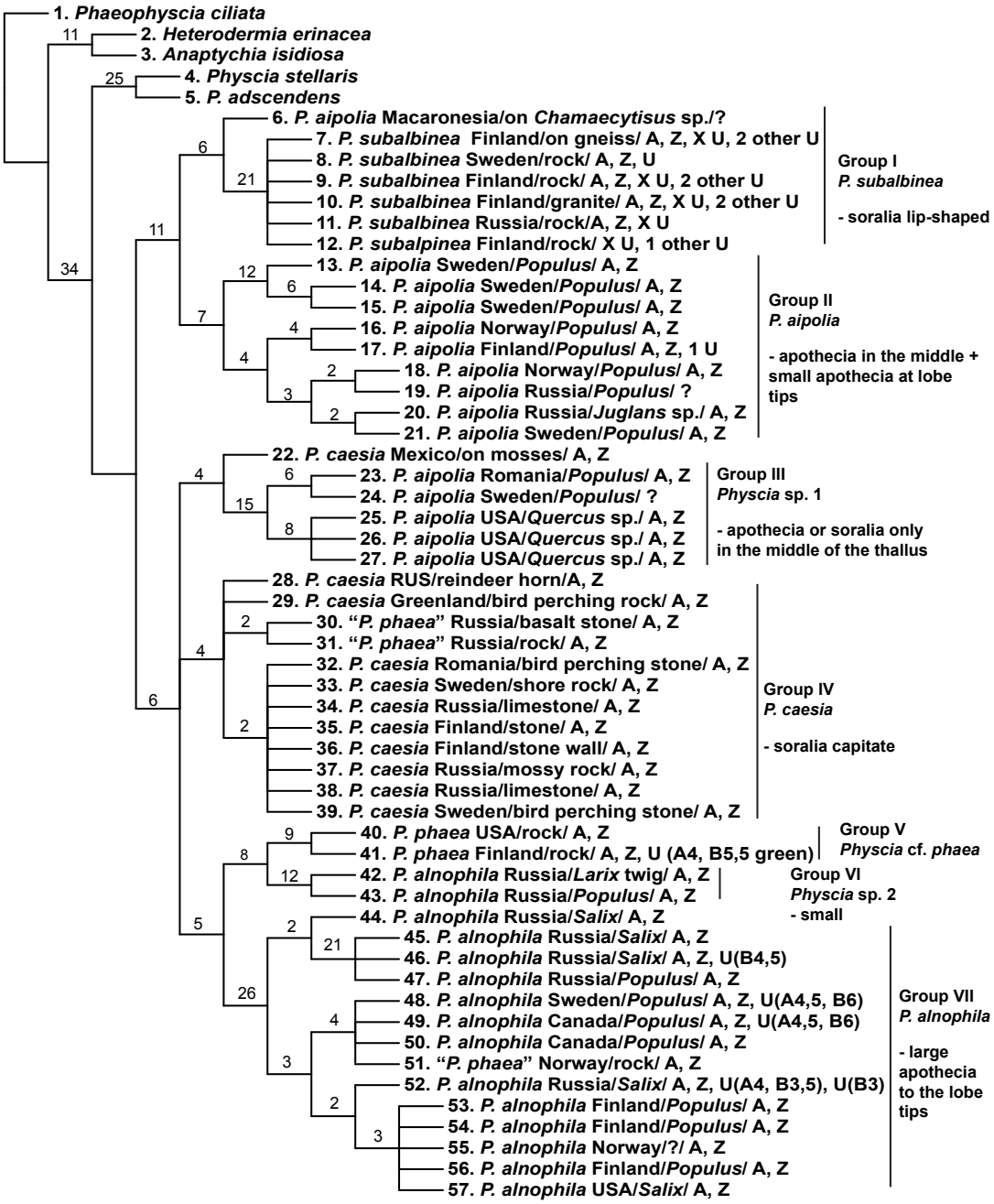

Fig. 1. POY-tree based on combined mtSSU rDNA, ITS, *betatubulin* and group I intron data sets. The figures on each node indicate Bremer support. Location/substrate/secondary chemistry (A = atranorin, Z = zeorin, X = UV+ blue unknown, U = other unknown substance.

all but one specimen (specimen 20 from walnut) in this group have been collected on *Populus*. One of the specimens (19) was not available for TLC, but all the other specimens contain atranorin and zeorin. Specimen 17 also contains the substance "X-unknown" (Rf A 2, UV+ blue) that is otherwise present in the *P. subalbinea* group (*see* above).

Group III: *Physcia* sp. 1

Group III consists of a basal *P. caesia* from Mexico and a well-supported group (Bremer support 15) of *P. aipolia* specimens mainly from the U.S.A., but also from Romania (specimen 25) and Sweden (specimen 26). The European and American specimens group separately. The specimens in this group differ from the rest of the *P. aipolia* and *P. caesia* specimens by having crowded apothecia/soralia at the middle of the thallus and a very wide peripheral thallus margin without any dispersal organs. The specimens in this group only contain atranorin and zeorin. Specimen 24 was unfortunately not available for TLC at the moment. Group III may represent a distinct species.

Group IV: *Physcia caesia*

Group IV (Fig. 1) contains *P. caesia* specimens that have capitate soralia (hence typical *P. caesia*). The group has a rather poor Bremer support (only 4) and contains specimens from Greenland, Finland, Romania, Russia and Sweden. Most of the specimens were collected on bird-perching stones, limestone or stone wall (*see* Fig. 1). All specimens in this group contain atranorin and zeorin.

Group V: *Physcia* cf. *phaea*

Group V (Fig. 1) consists of specimens collected in Finland and the U.S.A. Saxicolous specimens 40 and 41 were determined as *P. phaea*. The specimens contain atranorin and zeorin and specimen 41 also contains an unknown substance that was only visible in solvent A (Rf value 4).

Group VI: *Physcia* sp. 2

Specimens 42 and 43 were previously determined as *P. aipolia* var. *alnophila* and collected on trees (*Larix* and *Populus*, respectively). The thalli of these specimens appeared as much smaller than those of the average *P. aipolia* var. *alnophila* specimens. The specimens contained atranorin and zeorin. This group may represent an undescribed species.

Group VII: *Physcia aipolia* var. *alnophila*

Group VII consisted of typical *P. aipolia var. alnophila* specimens, having crowded small apothecia that occur at the tips of the thallus lobes. However, one of the specimens (51: Fig. 1) was determined as *P. phaea*, since it was collected on rock. The geographical origins of the specimens were Canada, Finland, Norway, Russia, and the U.S.A. Most of the specimens were from *Populus*, but nearly as many from *Salix*. This clade was further divided into several well-supported subgroups (*see* Fig. 1). All specimens contain atranorin and zeorin, but there are also some additional substances present. Specimen 46 contains an unknown substance that was only visible in the solvent B (Rf value 4.5). Specimens 48 and 49 contain two unknown substances with Rf values of 4.5 (A) and 6 (solvent B).

Since morphological characters, such as small thalli, as well as numerous, crowded, small apothecia that occur almost at the tips of the lobes (*see* Moberg 1977), together with the DNA sequence data and distinct distribution, uniformly support a recognition of an exclusive group with *Physcia aipolia* var. *alnophila* specimens, we here raise this taxon to species level.

Physcia alnophila (Vain.) Loht., Moberg, Myllys & Tehler, *comb. nova*

Physcia aipolia f. *alnophila* Vain 1881: 136. — *Physcia aipolia* var. *alnophila* (Vain.) Lynge 1916: 34. — LECTOTYPE (Moberg 1977): Finland. Li, Inari, Veskoniemi, 1878 *Vainio* (TUR-V 8070).

Thallus orbicular, up to 4 cm in diameter, with narrow lobes. Apothecia very common, crowded and small (up to 1.5 mm) usually with convex disc provided with white pruina. Spores 16–22(–24) × 7–10 μm.

In Scandinavia and Finland *Physcia alnophila* has a more northern distribution than *P. aipolia*, and it is often growing on *Alnus* and *Salix*, while *P. aipolia* prefers *Populus* (Moberg 1977). When collecting specimens for our study, *P. aipolia* and

P. alnophila were found growing side by side on *Populus* as far south as in central Sweden.

Discussion

There was a significant incongruence between the gene regions when the phylogenetic trees based on the different data sets were visually compared. Especially the nuclear and mitochondrial genes resulted in different tree topologies. The terminal groups were more or less stable in each gene tree, but the deep nodes were different between the nuclear and the mitochondrial gene trees. The addition of the mtSSU data to the rest of the data sets resulted in a tree that was different as compared with that in the study of Myllys *et al*. (2001).

We already knew from the study of Myllys *et al*. (2001) that the betatubulin, ITS and intron data sets were significantly incongruent, although there were only few discrepancies between trees based on the different data sets, incongruence in gene trees may be due to occasional gene flow and recombination of different taxa. However, Myllys *et al*. (2001) combined their data sets, since the combination of seemingly deviating data sets may reveal groupings that cannot be seen in any of the trees based on separate data sets. Also in the present case (Fig. 1) the analysis of the combined data set resulted in groups that had better support than in any of the trees based on individual data sets. Furthermore, new groups that were not present in trees based on individual data sets appeared in a tree based on the combined data set. However, more information of the specimens is needed to clarify whether hybridization, introgression or lineage sorting resulting from recent diversification is responsible for the deviating results.

Physciaceae is particularly rich in rDNA group I introns that are characterized by a conserved RNA primary and secondary structures (*see* Battacharya *et al*. 2002, Simon *et al*. 2005). For example Simon *et al*. (2005) observed extensive rDNA heterogeneity within *Physcia aipolia* and *P. stellaris* individuals. Simon *et al*. (2005) found different ITS sequences from single lichen thalli. We also found ITS PCR products of variable length from single DNA extractions, but when amplified with primers that omit intron at the end of the SSUrDNA region (*see* above), we obtained a single unambiquous DNA-sequence. The possible horizontal transfer of introns may, according to Hibbet (1996), make them unreliable source of information in phylogenetic studies. When we analyzed the ITS and the intron data sets separately (trees not shown), we found the topology of the resulting trees similar, which suggests that the introns and the ITS sequences provided rather congruent information.

Almost all groups except V and VI (Fig. 1) already existed in the study of Myllys *et al*. (2001). However, in that study (Myllys *et al*. 2001) specimens corresponding to *P. subalbinea* in the current study group together with the *Physcia* sp. 1 specimens (group III; Fig. 1). The difference is probably due to the precence of the mtSSU rDNA data in the current study.

Our separate study (unpubl. data) on the phylogeny of the genus *Physcia* indicates that only *P. aipolia*, *P. alnophila*, *P. caesia*, *P. phaea*, and *P. subalbinea* grouped in the *P. aipolia–P. caesia* group (sect. *Caesiae*; tree not shown). The rest of the species previously included in the sect. *Caesiae* (e.g. *P. poncinsii*, *P. rolfii, P. sinuosa*, *P. tribacoides*, *P. undulata* and *P. verrucosa*) are not closely related to this group.

Group I: *Physcia subalbinea*

Physcia subalbinea has a secondary chemistry different from most of the other specimens in this study. Some substances present in *P. subalbinea* were also found from specimens that group outside the *P. subalbinea* clade. Several TLC-tested *P. subalbinea* specimens not included into the molecular study had the substance "X-unknown", while the substance was absent from the tested *P. caesia* specimens. According to the molecular data (*see* Fig. 1) *P. subalbinea* is more closely related to *P. aipolia* than to another sorediate species *P. caesia* and thus represents a taxon distinct from *P. caesia*.

Group II: *Physcia aipolia*

Group II (Fig. 1) corresponds to typical *P. aipolia* based on morphological characters.

Group III: *Physcia* sp. 1

This clade contains apothecial and sorediate specimens. They all have a common feature with crowded apothecia/soredia only at the middle of the thallus and hence very wide thallus margins. The group containing the apotheciate specimens is very well supported. This clade (at least the well-supported group with the apotheciate specimens) most probably represents a species of its own.

Group IV: *Physcia caesia*

Group IV (Fig. 1) corresponds to *P. caesia*. Two of the specimens (30 and 31) had apothecia together with soralia and they were previously determined as *P. phaea*. This group is, however, poorly supported.

Group V: *Physcia* cf. *phaea*

All the apothecial specimens growing on rocks that were included in this study had been previously determined as *P. phaea*. However, only two of all these specimens (group V; Fig. 1) studied may actually correspond to *P. phaea*. The other "*P. phaea*" specimens appeared to be either *P. alnophila* growing on rocks or apotheciate *P. caesia* specimens. Specimens incorrectly determined as *P. phaea* have also been found from clades outside the *P. aipolia–P. caesia* group (authors' unpubl. data). Species delimitation and identification of this taxon is still somewhat unclear.

Group VI: *Physcia* sp. 2

Group VI consists of two specimens that have conspicuously minute thalli compared to the rest of the material (*see* Introduction). Since the specimens in this group (Fig. 1) appeared much smaller than those of *P. alnophila*, and they formed a separate well supported group (Bremer support 12), they may prove to represent a species new to science. However, more specimens are needed before the species status can be confirmed.

Group VII: *Physcia alnophila*

Physcia aipolia var. *alnophila* constituted a well-defined group that was clearly separate from *P. aipolia* var. *aipolia*, which instead grouped together with *P. caesia* (Fig. 1). Since the molecular data was also supported by morphological and ecological characters, we find that the new combination *P. alnophila* is well justified.

Species pairs

Based on this study it seems apparent that *P. aipolia* and *P. caesia* do not form a species pair *sensu* Poelt (1972), since the two taxa are more closely related to other species than to each other (Fig. 1). However, they seem not to form a single species either, as was suggested in Lohtander *et al*. (2000) and in Myllys *et al*. (2001), neither do they represent typical cryptic species (*see* e.g. Kroken & Taylor 2001), since the groupings are also supported by morphological and/or chemical characters. Instead, according to the molecular data, the *P. aipolia–P. caesia* group contains several groups that also have morphological and/or chemical characters and that thus can be treated as species.

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References

Battacharya, D., Friedl, T. & Helms, G. 2002: Vertical evolution and intragenic spread of lichen–fungal group I introns. — *Journal of Molecular Evolution* 55: 74–84.

Culberson, C. F. 1972: Improved conditions and new data for identification of lichen products by standardized thin layer chromatographic method. — *Journal of Chromatography* 72: 113–125.

Gardes, M. & Bruns, T. D. 1993: ITS primers with enhanced specificity for basidiomycetes — application to the iden-

tification of mycorrhizae and rusts. — *Molecular Ecology* 2: 113–118.

Gladstein, D. S. & Wheeler, W. 1997: *POY: the optimization of alignment characters. Program and documentation*. — American Museum of Natural History, New York.

Grube, M. & Arup, U. 2001: Molecular and morphological evolution in the Physciaceae (Lecanorales, lichenized Ascomycetes), with special emphasis on the genus *Rinodina*. — *Lichenologist* 33: 63–72.

Helms, G., Friedl, T. & Rambold, G. 2003: Phylogenetic relationships of the Physciaceae inferred from rDNA sequence data and selected phenotypic characters. — *Mycologia* 95: 1078–1099.

Hibbett, D. S. 1996: Phylogenetic evidence for horizontal transmission of group I introns in nuclear ribosomal DNA of mushroom-forming fungi. — *Molecular Biology and Evolution* 13: 903–917.

Kroken, S. & Taylor, J. W. 2001: A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. — *Mycologia* 93: 38–53.

Lohtander, K., Källersjö, M. & Tehler, A. 1998: Dispersal strategies in *Roccellina capensis* (Arthoniales). — *Lichenologist* 30: 341–350.

Lohtander, K., Källersjö, M., Moberg, R. & Tehler, A. 2000: The family Physciaceae in Fennoscandia: phylogeny inferred from ITS sequences. — *Mycologia* 92: 728–735.

Lohtander, K., Oksanen, I. & Rikkinen, J. 2002: A phylogenetic study of *Nephroma* (lichen-forming Ascomycota). — *Mycological Research* 106: 777–787.

Moberg, R. 1977: The lichen genus *Physcia* and allied genera in Fennoscandia. — *Symbolae Bot. Upsal.* 22(1): 1–108.

Moberg, R 1986: The genus *Physcia* in East Africa. — *Nordic Journal of Botany* 6: 843–864.

Moberg, R. 1990: The lichen genus *Physcia* in Central and South America. — *Nordic Journal of Botany* 10: 319–342.

Moberg, R. 1994: Is the Pacific a centre of speciation for some foliose genera of the lichen family Physciaceae. — *Journal of Hattori Botanical Laboratory* 76: 173–181.

Moberg, R. 2002: *Physcia*. — *Nordic Lichen Flora* 2: 33–38.

Myllys, L., Lohtander, K., Källersjö, M. & Tehler, A. 1999: Applicability of ITS data in Arthoniales phylogeny. — *Lichenologist* 31: 461–476.

Myllys, L., Lohtander, K. & Tehler, A. 2001: Beta-tubulin, ITS and group I intron sequences challenge the species pair concept in *Physcia aipolia* and *P. caesia*. — *Mycologia* 93: 335–343.

Poelt, J. 1970: Das Konzept der Artenpaare bei den Flechten. — *Vortr. Gesamtgeb. Bot.* N. F. 4: 187–198.

Poelt, J. 1972: Die Taxonomische Behandlung von Artenpaaren bei den Flechten. — *Botaniska Notiser* 125: 77–81.

Simon, D. M., Hummel, C. L., Sheeley, S. L. & Bhattacharya, D. 2005: Heterogeneity of intron presence or absence in rDNA genes of the lichen species *Physcia aipolia* and *P. stellaris*. — *Current Genetics* 47: 389–399.

Swofford, D. L. 2000: PAUP*. *Phylogenetic analysis using parsimony (*and Other Methods)*, ver. 4. — Sinauer Associates, Sunderland, Massachusetts.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap-penalties and weigh matrix choice. — *Nucleic Acids Research* 22: 4673–4680.

Wheeler, W. 1996: Optimization alignment: the end of multiple sequence alignment in phylogenetics? — *Cladistics* 12: 1–9.

White, T. J., Bruns, T., Lee, S. B. & Taylor, J. W., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (eds.), *PCR Protocols: a guide to methods and applications*: 315–322. Academic Press, Inc., New York.

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