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Photobiont Diversity in the Physciaceae (Lecanorales)

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Abstract. *Newly designed algal-specific primers were used to amplify the ribosomal ITS region from 25 photobiont specimens from five lichenized fungi of the family Physciaceae (Lecanorales),* Anaptychia ciliaris, Phaeophyscia orbicularis, Physcia caesia, P. tenella, *and* Physconia distorta. *The obtained DNA sequences were then phylogenetically analyzed using parsimony jackknifing. The analyses indicated that the mycobionts associated with two photobiont species:* Trebouxia impressa *was found with all mycobionts, except* Anaptychia ciliaris, *which instead was associated with* Trebouxia arboricola. *In the jackknife tree, all* Trebouxia arboricola *sequences formed a monophyletic group with a high jackknife support. The* Trebouxia impressa *sequences also formed a well supported group, which in turn had some internal structure. The photobiont species is reported for the first time for* Anaptychia ciliaris, Physcia caesia, *and* P. tenella. *A phylogenetic tree for the photobiont,* T. impressa, *was compared to a phylogeny of the corresponding mycobionts, also based on ITS sequences. A combined analysis of the data from the photobiont and the data from the mycobiont was also performed. Several similarities were found in the tree topologies. The general similarity of the mycobiont and photobiont trees may indicate a coevolutionary history.*

Lichens consist of two components, the mycobiont (a fungus) and the photobiont (a green alga and/or a cyanobacterium). The two live in symbiosis and can be said to constitute a small ecosystem. Since the mycobiont and the photobiont live in a close relationship it has been suggested that they coevolve.

The first to use the concept coevolution in a scientific paper was Ehrlich and Raven (1964). Since then there have been several new definitions of the word. Janzen (1980) defined it as "an evolutionary change of the individuals in one population in response to a trait in a second population, followed by an evolutionary response by the second population to the change in the first." Futuyma (1998) described coevolution as "reciprocal genetic change in interacting species owing to natural selection imposed by each on the other." For further information on coevolution and definitions see Thompson (1994) and the extensive references therein.

The concept can be used in a restricted sense, requiring synchronous speciation events of different taxa, co-speciation. It may also be used in a more relaxed sense. Parallel cladogenes (Futuyma 1998) concerns correlated evolution along lineages, but need not involve concomitant events of speciation. Specialization means constraints in the number of other taxa with which a particular species interacts. In lichens this is often referred to as specificity i.e., the tendency for a mycobiont to associate with a specific photobiont. The specificity may vary in different groups. Within some genera of fungi e.g., *Chaenotheca,* several algal genera are found as photobionts (Tibell 1984). Other fungal genera are always associated with a certain photobiont genus or even a specific species. At the species level, some mycobionts e.g., *Xanthoria parietina,* are affiliated with several photobiont species, and others e.g., *Usnea filipendula,* only with one (Ahmadjian 1993).

Ahmadjian (1987) was one of the first to suggest that lichen bionts coevolve. He argued that the coevolution in lichens might even be so far advanced that the photobiont and the mycobiont have become completely dependent of each other. He used the

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algal genus *Trebouxia* as an example to support this idea, since this genus has long been thought to be obligately lichenized. It should be noted, however, that there are a few reports of free-living *Trebouxia* (Mukhtar et al. 1994; Tschemak-Woess 1978).

Systematic studies involving lichens have so far focused on the mycobiont, whereas the photobiont has received less attention. For most mycobionts it is still unknown with which photobiont species they are affiliated. One of the reasons for this is that it has been virtually impossible to identify the photobiont species without culturing (Friedl & Rokitta 1997). However, in recent years a new tool for identifying species has become available through the use of molecular techniques such as DNA sequencing.

This study has two aims, the first is to identify the photobiont species from five lichenized fungi of the family Physciaceae (Lecanorales), *Anaptychia ciliaris, Phaeophyscia orbicularis, Physcia caesia, P. tenella,* and *Physconia distorta,* using sequences from the ITS region of the nuclear ribosomal repeat. The second aim is to use these sequences to construct a phylogeny for the photobionts and compare it to a phylogeny of the corresponding mycobionts, the latter based on ITS sequences from Lohtander et al. (2000) in order to investigate a possible common evolutionary history.

Materials and Methods

Taxon sampling.—The selection of photobionts was done so to represent the three major clades of foliose Physciaceae (Lohtander et al. 2000): *Physcia caesia* and *P. tenella* were chosen from the *Physcia*-group, *Phaeophyscia orbicularis* from the *Phaeophyscia*-group, and *Physconia distorta* and *Anaptychia ciliaris* from the *Physconia-group.* Each species was represented by five specimens, collected from different substrates and different geographical locations, mostly in Sweden and Finland (Table 1). Vouchers are deposited at the Swedish Museum of Natural History (S), or at the Museum of Evolution at Uppsala University (UPS). The ITS region, including ITS1, 5.8S, ITS2 from the nuclear, ribosomal repeat (Fig. 1), was sequenced for the photobionts of all lichens. For 18 specimens the same DNA extract was used as in Lohtander et al. (2000). New sequences have been submitted to GenBank (Table 1). In addition, 19 algal ITS sequences were retrieved from GenBank (Table 2). The mycobionts were represented by 18 ITS sequences taken from Lohtander et al. (2000).

DNA extraction.—Extractions of DNA were performed using QIAGEN's QIAamp DNA Mini Kit following the manufacturers instructions with slight modifications: The tissue (a small piece of thallus) was incubated in 56°C overnight in a stationary water bath, steps 7a and 9 were not performed, and the DNA was eluted in 50 µl elution buffer.

Primers.—All primers are listed in Table 3. New, algal specific PCR primers, ITS1AKL and ITS6AKL, were designed by comparing the 3′ end of 18S and the 5′ end of 26S from several green algal and fungal sequences in GenBank (Fig. 1) (Chlorophycean algae AF23398, *Scenedesmus pupukensis* X91267 and *Chlamydomonas nivalis* U57696 for ITS1AKL, and *Pandorina morum* U23533 and *Chlorella ellipsoidea* D17810 for ITS6AKL). The sequencing primer ITS2AD, located at the 3′ end of the ITS 2 region, was based on the ITS region of *Trebouxia impressa* in GenBank (Acc. No. AJ007388). For the photobiont of *Anaptychia ciliaris* an alternative primer, ITS4AD, was designed, based on sequences from the photobiont of specimen 301 (Table 1).

PCR.—A total DNA extraction, including fungal as well as algal DNA was used as a template. Amplification was performed using Pharmacia Biotech's Ready To Go PCR Beads, according to the manufacturers instructions. The cycling profile was 95°C–one min., 50°C–one min., and 72°C–one min., repeated 30 times. The products were purified with QIAGEN's QIAquick PCR purification Kit following the manufacturers protocol, except that 44 µl elution buffer was used.

Sequencing.—The Cy5 labeled primers ITS1LM, ITS2AD, and ITS4AD were used for sequencing (Table 3). The reactions were performed using Amersham's ThermoSequenase sequencing kit, starting with denaturation for two min. in 95°C, followed by a 30-cycle with settings of 95°C–30 sec., 50°C–30 sec., and 72°C–one min. The samples were electrophoresed on a 6% Long Ranger gel in a Pharmacia Biotech Inc. ALFExpress automatic sequencer.

Phylogenetic analysis.—Three sets of sequences were selected to construct alternatively aligned data matrices. For the purpose of identifying the photobionts, an extensive photobiont set (Tables 1, 2) was constructed, including all available *Trebouxia* sequences in GenBank. For comparisons of algal and fungal phylogenies, two sets including only specimens within the same lichen associations were constructed: a reduced photobiont set and a corresponding mycobiont set based on sequences from Lohtander et al. (2000). The sequences from the mycobiont and the reduced photobiont sets were also combined into a single matrix.

All data sets were aligned using ClustalW 1.5 (Thompson et al. 1994). *Trebouxia gigantea* was removed from the extensive photobiont set because of several large insertions that made alignment difficult. For the same reason a unique insertion of 75 base pairs in *Trebouxia gelatinosa* was identified and excluded. Several alignments were made with gap opening penalties between 2–20 and gap extension penalties between 1–5. Alternative alignments were also made for the reduced photobiont set and the mycobiont set. Phylogenetic analyses were performed with the parsimony jackknifing program "Xac" (Farris 1997), using a version of the program that allows branch swapping and random taxon addition. The following settings were used: 1,000 replicates with branch-swapping and five random addition sequences each. Gaps were coded in two ways, as a missing datum or as a fifth character state. Phylograms were obtained using PAUP 4.0b3 (Swofford 2000), with the following settings: heuristic search, TBR, multrees off, and 1,000 random stepwise additions. Trees were rooted using the outgroup criterion (Farris 1972). *Chlorella ellipsoidea* was used as an outgroup for the extensive photobiont set, whereas *Anaptychia ciliaris* and its photobiont were used for the mycobiont and reduced photobiont set trees, respectively and also for the combined analysis. The reason for the latter choice is that it was the only photobiont, outside *Trebouxia impressa,* from which we had sequences also of the corresponding mycobiont. That made the mycobiont tree differently rooted compared to that of Lohtander et

TABLE 1. Lichen Specimens. Substrate, mycobiont, and GenBank number listed.

Collection area	Substrate	Collector	Deposition place	GenBank number	
				Photobiont	Mycobiont
Anaptychia ciliaris (L.) Körb.					
Sweden, Uppland, Bogesund	*Acer platanoides*	*Dahlkild 6*	(S)	(AF389916)	
Sweden, Uppland, Tullgarn	*Fraxinus excelsior*	*Lohtander 498*	(S)	(AF389914)	(AF224366)
Sweden, Uppland, Velamsund	*Acer platanoides*	*Lohtander 301*	(S)	(AF389913)	(AF224365)
Sweden, Uppland, Östanå	*Quercus robur*	*Dahlkild 11*	(S)	(AF389915)	
Sweden, Uppland, Östra Ryd	*Tilia cordata*	*Dahlkild 9*	(S)	(AF389917)	
Phaeophyscia orbicularis (Neck.) Moberg					
Finland, Nylandia, Tammela	*Fraxinus excelsior*	*Lohtander & Jalonen 342*	(S)	(AF389929)	(AF224451)
Finland, Region aboënsis, Angelniemi	*Populus tremula*	*Myllys & Kuusinen 25*	(S)	(AF389931)	(AF389939)
Sweden, Uppland, Estuna	*Populus tremula*	*Tehler 7901*	(S)	(AF389932)	(AF389938)
Sweden, Uppland, Uppsala	*Ulmus glabra*	*Moberg 12018*	(UPS)	(AF389928)	(AF224459)
Sweden, Uppland, Väddö	*Populus tremula*	*Tehler 7893*	(S)	(AF389930)	(AF334356)
Physcia caesia (Hoffm.) Fürnr.					
Finland, Alandia (Åland), Lemland	rocks	*Tehler 7934*	(S)	(AF389922)	(AF224386)
Finland, Nylandia, Helsinki	rocks	*Lohtander 346*	(S)	(AF389921)	(AF224389)
Mexico, E. D. Mexico, Nevado de Toluca	rocks	*Nordin 3847*	(UPS)	(AF389920)	(AF224438)
Russia, Siberia, Kolyma delta	rocks	*Mattson 3341*	(UPS)	(AF389919)	(AF224436)
Sweden, Uppland, Uppsala	rocks	*Moberg 12019*	(UPS)	(AF389918)	(AF224384)
Physcia tenella var tenella (Scop.) DC.					
Finland, Nylandia, Sipoo	*Quercus robur*	*Lohtander & Jalonen 311*	(S)	(AF389935)	(AF224425)
Finland, Nylandia, Siuntio	*Populus tremula*	1995 *Myllys s.n.* (M12)	(S)	(AF389937)	(AF224427)
Finland, Regio aboënsis, Karjalohja	*Populus tremula*	*Lohtander & Jalonen 596*	(S)	(AF389934)	(AF389940)
Sweden, Sörmland, Tullgarn	*Tilia cordata*	*Lohtander 496*	(S)	(AF389933)	(AF389941)
Sweden, Uppland, Uppsala	*Quercus robur*	*Dahlkild 15*	(S)	(AF389936)	
Physconia distorta (With.) J.R. Laundon					
Finland, Nylandia, Sipoo	*Quercus robur*	*Lohtander & Jalonen 313*	(S)	(AF389924)	(AF224373)
Sweden, Gotland, Östergarn	*Fraxinus excelsior*	*Moberg 12036*	(UPS)	(AF389923)	(AF224371)
Sweden, Uppland, Bogesund	*Quercus robur*	*Dahlkild 3*	(S)	(AF389926)	
Sweden, Uppland, Östanå	*Fraxinus excelsior*	*Dahlkild 7*	(S)	(AF389927)	
Sweden, Uppland, Östra Ryd	*Tilia cordata*	*Dahlkild 10*	(S)	(AF389925)	

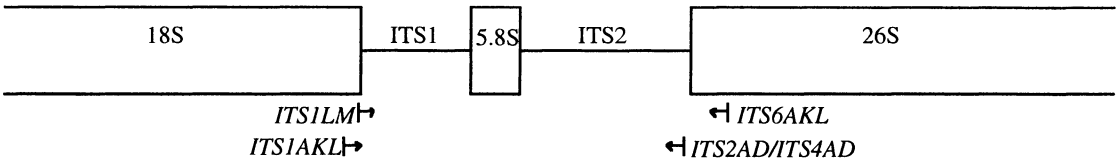


FIGURE 1. Primer position in the ITS region.

al. (2000) that included a larger number of taxa. Alignments and trees are available at TreeBASE.

RESULTS

Extensive photobiont set.—Comparisons between the newly sequenced algae and sequences from GenBank confirmed that we had sequenced the algal ITS region. Two of the new sequences, from lichen specimens *Physcia tenella* D15 and *P. tenella* 596 were found to be identical and could indicate possible contamination. However, we do not believe this to be the case, since *Physcia tenella* D15 was collected and sequenced at a separate occasion from all other specimens. Also, we found that some of our new sequences were identical to GenBank sequences e.g., the photobiont from *Phaeophyscia orbicularis* 7901 is indistinguishable from *Trebouxia impressa* (Parmelia), Acc. No. AJ249570.

The algal sequences were 503–614 base pairs long. Alignments under different parameters showed only small differences and jackknife trees resulting from them were largely congruent, differing only in support frequencies and/or in degree of resolution. Figure 2 depicts a jackknife tree based on an alignment with gap opening penalty/gap extension penalty, 4/1 and an analysis where gaps are treated as missing data. The data set had 770 characters (223 informative). Below, and in Figure 2, Phb and their associate mycobiont name and collection number will refer to the new photobiont sequences, whereas GenBank sequences are referred to as algal species with their mycobiont associate in parenthesis. The tree shows that *Trebouxia* is divided into two monophyletic groups; 1) Group A, with *Trebouxia jamesii, T. corticola, T. usneae, T. galapagensis, T. higginsiae, T. asymmerica, T. arboricola* and all photobionts from *Anaptychia ciliaris,* and 2) *T. gelatinosa* together with Group C i.e., *T. impressa* from GenBank and the photobionts from *Phaeophyscia orbicularis, Physcia caesia, P. tenella,* and *Physconia distorta.*

Group A has support of 99% and is further divided into two well supported clades: one includes *Trebouxia jamesii, T. corticola, T. usneae, T. galapagensis,* and *T. higginsiae;* the other comprises *T. asymmetica, T. arboricola,* as well as the photobionts from the five *Anaptychia ciliaris* specimens. *Trebouxia asymmetica* is basal in the latter and sister to a group supported at 100%, consisting of the photobionts of *Anaptychia ciliaris* together with five *Trebouxia arboricola* from GenBank (group B). Within group B, Phb *Anaptychia ciliaris* 498 is the sister taxon to all the other taxa. Then follows Phb *Anaptychia ciliaris* D6 and *Trebouxia arboricola* (*Pleurosticta acetabulum*) as the sister

TABLE 2. Algae specimens from GenBank. Mycobiont and GenBank number listed.

Algae	Mycobiont	GenBank nr.
Chlorella ellipsoidea Gerneck		D13340
Trebouxia arboricola De Puymaly	*Lecidella elaeochroma*	AJ007385
	Xanthoria parietina	AJ007387
	Punctelia subrudecta	AJ249564
	Pleurosticta acetabulum 1	AJ249481
	Pleurosticta acetabulum 2	AJ249482
T. assymetrica Friedl & Gärtner	*Diploschistes diacapsis*	AJ249565
T. corticola (Archibald) Gärtner		AJ249566
T. galapagensis (Hildreth & Ahmadjian) Gärtner	*Ramalina sp.*	AF249567
T. gelatinosa Ahmadjian		Z68698
T. higginsiae (Hildreth & Ahmadjian) Gärtner	*Buellia straminea*	AF249574
T. impressa Ahmadjian	*Physcia adscendens* 1	AF007383
	Physcia adscendens 2	AJ007384
	Phaeophyscia orbicularis	AJ007386
	Parmelina tiliacea	AJ007388
	Parmelia carporrhizans	AJ249570
	Melanelia glabra	AJ249576
T. jamesii (Hildreth & Ahmadjian) Gärtner	*Imshaugia placorodia*	Z68701
T. usneae (Hildreth & Ahmadjian) Gärtner	*Usnea filipendula*	AJ249573

TABLE 3. Primer sequences. All primers are written in the 5′–>3′ direction. A = algal specific, U = universal, F = forward, R = reverse.

ITS1AKL	GTGCTGGTGAAGTGTTCGGA	A	F	PCR	(this study)
ITS6AKL	ATCTTGCCTGAGCTCAGG	A	R	PCP	(this study)
ITS1LM	GAACCTGCGGAAGGATCATT	U	F	Sequencing	(Myllys et al. 1999)
ITS4AD	GGCGTCCTGCACAACGACTAC	A	R	Sequencing	(this study)
ITS2AD	GGCGTCCTGCACACGCTTTC	A	R	Sequencing	(this study)

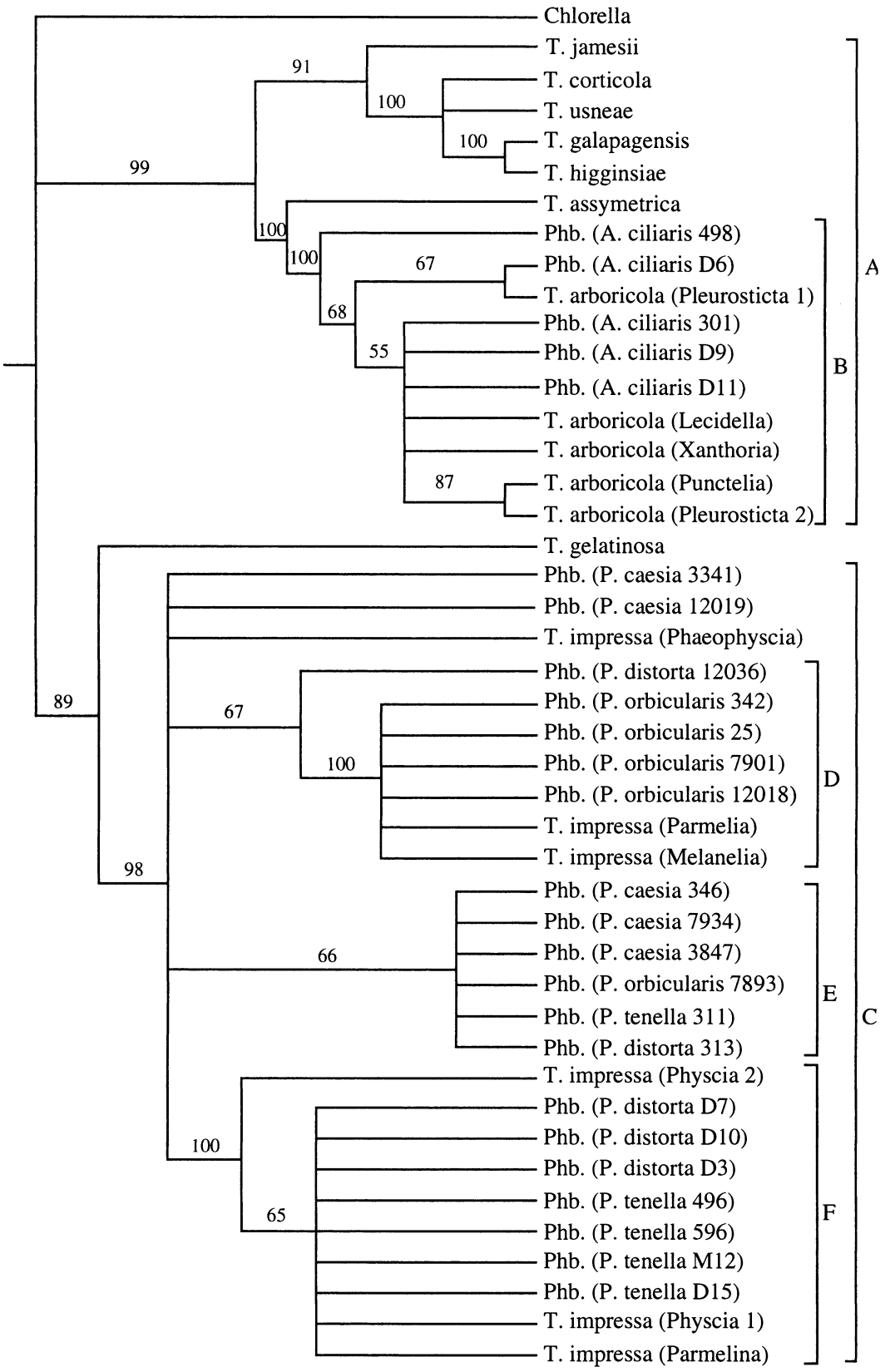
group to the remainder. Within the latter clade, there is a sister pair with *Trebouxia arboricola* from *Punctelia subrudecta* and *Pleurosticta acetabulum.* In the other major *Trebouxia* group, *T. gelatinosa* is sister to Group C. Within group C there is a basal hexatomy. Three of its branches consist of single terminals, the photobionts of *Physcia caesia* 3341 and 12019 and *T. impressa* (*Phaeophyscia*) from GenBank. A fourth branch (group D) has low support of 67% and includes Phb *Physconia distorta* 12036 in a basal position as sister to a group, supported at 100%, consisting of four specimens from the photobionts of *Phaeophyscia orbicularis,* together with two *T. impressa,* (*Parmelia, Melanelia*). A fifth branch (group E), supported at 66%, consists of three photobionts from *Physcia caesia* together with one specimen of the photobiont from *Phaeophyscia orbicularis,* one from *Physcia tenella,* as well as one photobiont from Physconia *distorta.* A sixth branch of the hexatomy (group F) is formed by photobionts from three specimens of *Physconia distorta,* four from *Physcia tenella* and three specimens of *T. impressa* from GenBank, two from *Physcia adscendens* (*Physcia* 1, 2), and one from *Parmelina tiliacea.* Group F has support of 100% and within this clade there is only one supported group (65%) identifying the GenBank sequence of *T. impressa* (*Physcia* 2) as sister to the other taxa.

A strict consensus tree, calculated by PAUP*, resulting from a parsimony analysis of the same matrix as above, is presented in Figure 3 as a phylogram. The consensus tree is based on 760 most parsimonious trees with a length of 649 steps and is more resolved, but not in conflict with the jackknife tree.

Coding gaps as a fifth character state for the same data matrix as above, distinctly increased the number of informative characters from 223 to 325. However, the different coding had little impact on overall tree topology (tree not shown). A few additional groups were found: a well supported (98%) group in group B formed by *Trebouxia arboricola* from *Xanthoria parietina* and *Lecidella elaeochroma*; Phb *Physcia caesia* 3341 and Phb *Physcia caesia* 12019 that are unresolved at the base of group C in the tree above, are here basal to other photobionts from *Physcia caesia* in group E. Also, the support for group E increases from 66% to 94%. One group, the association of Phb *Physconia distorta* 12036 to other taxa in group D, was lost compared to the tree in Figure 2. The only conflict between the two trees concerns group F. When gaps are treated as a fifth character state, *T. impressa* (*Physcia* 1) is found as sister to *T. impressa* (*Phaeophyscia*) in an otherwise unresolved group F. When gaps are coded as missing information (Fig. 2) *T. impressa* (*Phaeophyscia*) is found unresolved at the base of group C, whereas *T. impressa* (*Physcia* 2) is sister to all other taxa in group F.

Reduced photobiont set.—Different alignment parameters produced similar data sets. The jackknife tree in Figure 4 is based on an alignment with a gap opening penalty/gap extension penalty of 4/1, and an analysis with gaps treated as missing information. The data set had 650 characters (83 informative). The tree has some different grouping compared to the more extensively sampled analysis in Figure 2. The basal polytomy of group C is here resolved. Phb *Physconia distorta* 12036 is sister to all other *T. impressa.* (In the more extensive analysis it appeared basal in group D). It is followed by the other D group taxa, four photobionts from *Phaeophyscia orbicularis,* as sister to the remaining ingroup. The photobionts from *Physcia caesia* 12019 and *P. caesia* 3341 were unresolved in Group C in the extensively sampled analysis (Fig. 2). In the Reduced photobiont set tree (Fig. 4) the former appears as sister to group E (including photobionts from four mycobiont species, *Physconia distorta* 313, *Phaeophyscia orbicularis* 7893, *Physcia tenella* 311, and *P. caesia* 7934, 346, 3847). The latter, Phb *Physcia caesia* 3341, is sister to groups E and F (three photobionts from *Physcia tenella*) and Phb *Physcia caesia* 12019. Support for these new groupings is low (71%), with the exception of the association of Phb *Physcia caesia* 12019 to group E that is supported at 96%.

Mycobiont data set.—The data set has 602 characters (124 informative) and the tree is shown in Fig. 5. There is no conflict between this tree and that in Lohtander et al. (2000). *Physconia distorta* is sister to the remainder of the ingroup. The next clade to diverge consists of the five *Phaeophyscia orbicularis.* Within *Phaeophyscia orbicularis* there are two monophyletic groups, with terminals 12018


Chlorella
T. jamesii
T. corticola
T. usneae
T. galapagensis
T. higginsiae
T. assymetrica
Phb. (A. ciliaris 498)
Phb. (A. ciliaris D6)
T. arboricola (Pleurosticta 1)
Phb. (A. ciliaris 301)
Phb. (A. ciliaris D9)
Phb. (A. ciliaris D11)
T. arboricola (Lecidella)
T. arboricola (Xanthoria)
T. arboricola (Punctelia)
T. arboricola (Pleurosticta 2)
T. gelatinosa
Phb. (P. caesia 3341)
Phb. (P. caesia 12019)
T. impressa (Phaeophyscia)
Phb. (P. distorta 12036)
Phb. (P. orbicularis 342)
Phb. (P. orbicularis 25)
Phb. (P. orbicularis 7901)
Phb. (P. orbicularis 12018)
T. impressa (Parmelia)
T. impressa (Melanelia)
Phb. (P. caesia 346)
Phb. (P. caesia 7934)
Phb. (P. caesia 3847)
Phb. (P. orbicularis 7893)
Phb. (P. tenella 311)
Phb. (P. distorta 313)
T. impressa (Physcia 2)
Phb. (P. distorta D7)
Phb. (P. distorta D10)
Phb. (P. distorta D3)
Phb. (P. tenella 496)
Phb. (P. tenella 596)
Phb. (P. tenella M12)
Phb. (P. tenella D15)
T. impressa (Physcia 1)
T. impressa (Parmelina)
91
100
100
99
100
100
67
68
55
87
89
67
100
98
66
100
65
A
B
C
D
E
F

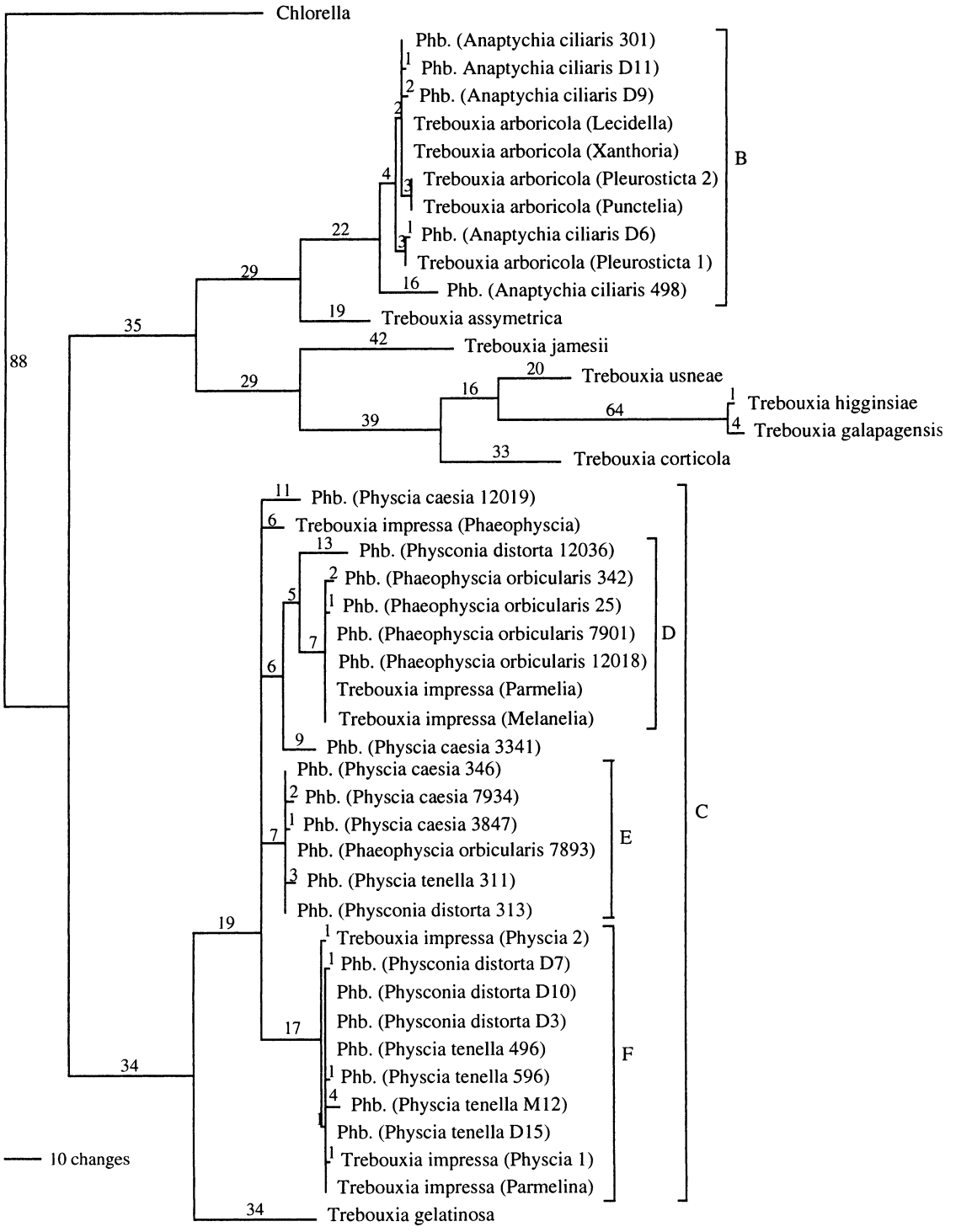


FIGURE 3. Phylogram obtained from strict consensus of 760 most parsimonious trees based on the *Extensive photobiont set.* Phb = Photobiont of named mycobiont.

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FIGURE 2. Jackknife tree obtained from the *Extensive photobiont set.* Jackknife frequencies are shown at nodes. Phb = Photobiont of named mycobiont.

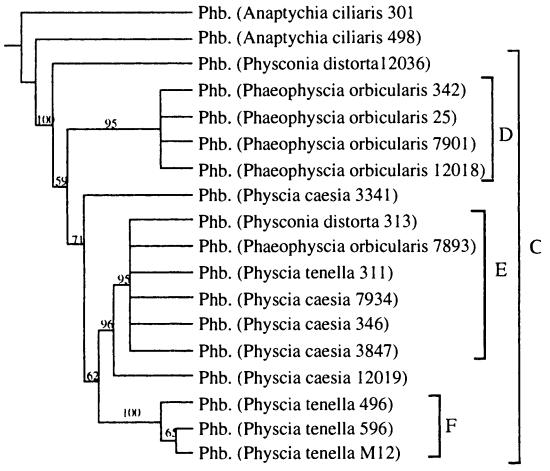

FIGURE 4. Jackknife tree obtained from the *Reduced photobiont set.* Jackknife frequencies are shown at nodes. Phb = Photobiont of named mycobiont.

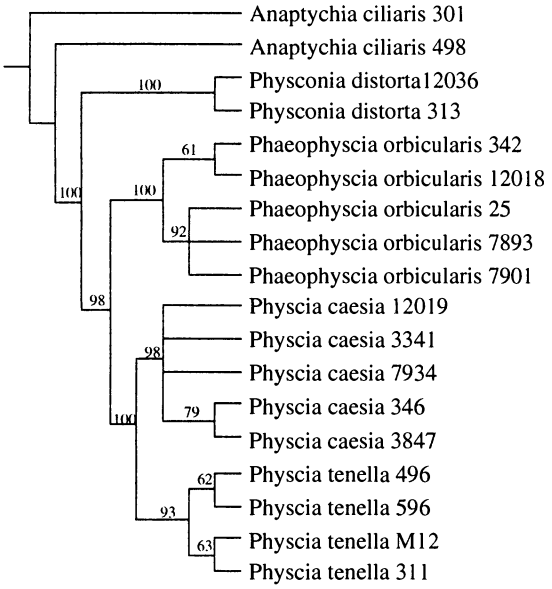

FIGURE 5. Jackknife tree obtained from the *Mycobiont data set.* Jackknife frequencies are shown at nodes.

and 342 in one clade and terminals 7893, 7901, and 25 in the other. The sister group to *Phaeophyscia orbicularis* is a clade including *Physcia tenella* and *Physcia caesia* as sister groups. Within the *Physcia tenella* group there is a dichotomy: one clade with terminals M12 and 311 and another with terminals 496 and 596. The *Physcia caesia* group is unresolved, except for a sister pair with terminals 346 and 3847. The four species, *Physcia caesia, Physcia tenella, Phaeophyscia orbicularis,* and *Physconia distorta,* form a monophyletic group with jackknife support of 100%.

Combined data set.—The data set has 1,252 characters (207 informative) and the tree is shown in Fig. 6. The tree *topology* is similar to the topology of the mycobiont tree with the following exceptions: The support for the *Physconia distorta* clade decreases from 100 to 87%. In the *Phaeophyscia orbicularis* clade, the group with terminals 7893, 7901, and 25 disappears, and instead terminal 7893 is found basal to all other *P. orbicularis.* In the *Physcia tenella* clade, terminal 311 becomes the sister group to all other *P. tenella* terminals, and the support for the clade with terminals 496 and 596 increases from 62% to 99%. The topology of the *Physcia caesia* clade is fully resolved. Terminal 3341 is found in a basal position followed by terminal 12019. The group with terminals 346 and 3847 receives a higher jackknife support (86% compared to 79%).

DISCUSSION

Identification of photobiont.—Most molecular studies of photobionts have involved culturing the algae, which is a time consuming process. This study shows that it is perfectly feasible to amplify both the mycobiont and the photobiont from the same extraction, using specific primers. The PCR primers ITS1AKL and ITS6AKL, designed for this study, should work for a broad range of green algae (within Chlorophyta), whereas the sequencing primers ITS2AD and ITS4AD are designed specifically for *Trebouxia impressa* and *Trebouxia arboricola,* respectively.

The algal ITS sequences had sufficient variation

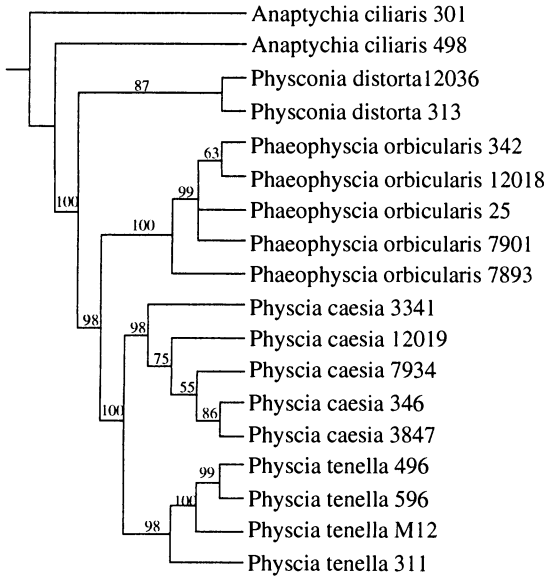

FIGURE 6. Jackknife tree of the combined *Reduced photobiont set* and the mycobiont data set. Jackknife frequencies are shown at nodes. Lichens represent terminals.

to provide supported resolution between and, to some extent, within species. The species identity of the new photobiont sequences could be determined since they were found nested with high support among known algal sequences from GenBank (Figs. 2, 3). All investigated specimens from *Phaeophyscia orbicularis, Physcia caesia, P. tenella,* and *Physconia distorta* were found to have *Trebouxia impressa* as a photobiont. This is in agreement with Beck et al. (1998) who reported that *Phaeophyscia orbicularis* and *Physcia adscendens* were associated with *Trebouxia impressa.* In contrast, the well supported group B (Fig. 2) indicates that *Anaptychia ciliaris* has strictly *Trebouxia arboricola* as its photobiont. If the association with *T. impressa* is assumed to be the ancestral condition in the Physciaceae, which is reasonable considering the phylogeny presented by Lohtander et al. (2000), the specificity of *Anaptychia ciliaris* to *T. arboricola* could represent a host switch. In Figure 2, photobionts from the Physciaceae and the Parmeliaceae are found intermixed, and *Trebouxia impressa* is obviously not restricted to the family Physciaceae.

Effects of outgroup selection on tree topology.—The choice of outgroups for the extensive photobiont set was difficult. Beck et al. (1998) stated that there was no suitable outgroup for analyzing *Trebouxia* ITS sequences. In a study of *Trebouxia,* based on 18S and 26S rDNA, Friedl and Rokitta (1997) included a larger selection of green algae. According to their results, *Chlorella saccharophila* or *Dictyochlapsis reticulata* could be a possible sister group to *Trebouxia.* Unfortunately, there are no ITS sequences available for either of these taxa in GenBank. The closest species we found was *Chlorella ellipsoidea,* but that sequence was only from 5.8S and ITS 2 so the ITS1 region had to be replaced with question marks in the data matrix. We made an exploratory phylogenetic analysis with *Trebouxia* species from GenBank and *Chlorella ellipsoidea* and found that the resulting tree topology was the same as in Friedl and Rokitta (1997, pages 136 and 138). Therefore we considered *Chlorella ellipsoidea* suitable as an outgroup for this study. However, it may be that *Chlorella ellipsoidea* is too distantly related to the ingroup. We therefore tried one of the ingroup taxa, *Trebouxia jamesii,* as the outgroup. The topology did not change with *T. jamesii* as the outgroup, but support for the various nodes decreased, and for this reason we decided to keep *Chlorella* as the outgroup.

As explained in the Materials and methods, photobionts from *Anaptychia ciliaris* were selected as outgroups for the reduced photobiont data. The resulting tree is similar overall to that found after analysis of the extensive data set, but the change of outgroup causes the ingroup to be effectively rerooted so that *Physconia distorta* 12036 is basal, followed by the other taxa in group D. Likewise, the use of *Anaptychia* as outgroup for the mycobiont tree causes the ingroup to be rerooted in comparison to the tree of Lohtander et al. (2000).

Comparisons of photobiont and mycobiont phylogenies.—A consensus tree constructed from the mycobiont and the photobiont trees is almost completely unresolved (not shown). However, when inspecting the individual trees (Figs. 4, 5) it is obvious that there are similarities in the basic structure that could be interpreted as a result of a common evolutionary history. Two groups in the photobiont tree include only algae associated with the same mycobiont species, *Phaeophyscia orbicularis* and *Physcia tenella,* respectively. Also, the sister group relation of *Physconia distorta* to the rest of the taxa can be found in the photobiont tree. There are, however, also some obvious differences between the two trees. All *Physcia caesia* photobionts but one are found in the same group, however, this group also includes terminals associated with a mix of mycobiont species. Also, if the specimens appearing in the 'mixed' group are removed from the tree the resulting taxa show a nearly perfect congruence with the mycobiont tree.

We also experimented with another approach to compare data. In lichenology, when using morphological data, it is not unusual to add information related to the photobiont when constructing phylogenies (Sundin & Tehler 1998; Tehler 1990; Thor 1990). Here, in a similar fashion, we combined molecular mycobiont data with molecular photobiont data in a single analysis. If there is a common history for the bionts an analysis of the combined data should result in increased support frequencies and retained or improved resolution. The opposite would be the expected if there were no such correlation.

The combination of data did not cause a loss of tree resolution, as would have been expected if there was a major conflict between the different kinds of data. In fact, the combined tree shows increased resolution, with 14 supported groups compared to 12 in the mycobiont tree and nine in the photobiont tree. The combined tree is similar to the mycobiont tree, showing monophyly for all mycobiont species. The increased support for the two sister pairs, *Physcia caesia* 346 and *Physcia caesia* 3847 and *Physcia tenella* 496 and *Physcia tenella* 596, in the combined analysis indicates that coevolution may have occurred. However, some character conflict is introduced causing the lichen specimens with photobionts from the 'mixed' group (mentioned above) to move into a basal position in each species.

The general similarity of the mycobiont and photobiont trees could indicate a common evolutionary history. However, it is also obvious that there is no perfect correlation between the biont trees and under a model of coevolution it becomes necessary to assume multiple host switches. Also, when comparing the reduced phylogenies to the extensive photobiont tree in Fig. 2, it is clear that *T. impressa* is by no means restricted to the Physciaceae, since photobiont sequences originating from the lichens *Parmelia* and *Melanelia* are interspersed among the Physciaceae photobionts.

Lichens that reproduce by asexual propagules, such as soredia, in which the mycobiont and photobiont disperse together, may be considered as clones (Tehler 1982). A mycobiont reproducing sexually has to associate with a photobiont in order to develop a new lichen thallus. This can be accomplished in different ways for example by finding a freeliving alga (Mukhatar et al. 1994) or by "stealing" a photobiont from an already established lichen (Beck 1999; Honegger 1993). *Physconia distorta* only reproduces sexually and that could explain its ability to switch photobiont lineage, since it needs to resynthesize the lichen on each dispersal event. The same explanation could be used for the atypical photobionts found in *Phaeophyscia orbicularis* and *Physcia tenella,* since both sometimes reproduce sexually.

The similarities in tree topologies could possibly be related to other factors, such as substrate preference, geographical location, or reproductive strategies. However, as can be seen in Table 1, we have found no obvious correlations between tree topology, geography, or substrate preference.

To further investigate possible correlations between the phylogenies, a more extended study of lichen photobionts and mycobionts should be made including more extensive sampling, both systematically and geographically, and also information from more than one gene region. It would also be interesting to perform an analysis with obligately sexually reproducing mycobionts together with obligately asexually reproducing mycobionts.

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