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Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies

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SUMMARY

Axenic cultures of lichen photobionts isolated from bark-inhabiting lichen thalli of the *Physcietum adscendentis* Ochsner were identified by light microscopy and sequence comparisons of internal transcribed spacer rDNAs to investigate principles of lichenization within a defined lichen sociological unit. The photobiont identity of eight lichen species is reported for the first time (photobiont species in square brackets): *Lecania cyrtella* (Ach.) Th. Fr. [*Trebouxia arboricola* Puym.], *Lecania naegelii* (Hepp) Diederich & v. d. Boom [*Dictyochloropsis symbiontica* Tscherm.-Woess], *Candelaria concolor* (Dicks) B. Stein [*Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner], *Candelariella* cf. *reflexa* (Nyl.) Lettau [*T. jamesii*], *Lecanora* spec. [*T. arboricola*], *Phaeophyscia orbicularis* (Neck.) Moberg [*T. impressa* Ahmadjian], *Physcia adscendens* (Fr.) H. Olivier [*T. impressa*] and *Lecidella elaeochroma* (Ach.) M. Choisy [*T. arboricola*] and could be confirmed for another two species, *Physcia stellaris* (L.) Nyl. [*Trebouxia impressa*] and *Xanthoria parietina* (L.) Th. Fr. [*Trebouxia arboricola*]. The observation that pioneer lichens without vegetative propagules, growing on smooth bark, had *Trebouxia arboricola* as photobiont can be explained by the assumption of a free-living population of *Trebouxia arboricola*. Species of photobionts from *Xanthoria parietina* were morphologically and genetically different from those of *Physcia adscendens* and *Phaeophyscia orbicularis*, respectively; a finding that does not support the previous assumption that *Xanthoria parietina* takes over its algal partner from a *Physcia* species, at least at the sites investigated.

Key words: Lichens, photobionts, mycobionts, photobiont choice, ITS ribosomal DNA.

INTRODUCTION

Lichens consist of at least one algal species (photobiont) and one fungal species (mycobiont). In the case that both bionts are distributed as separate units it is essential for their re-lichenization that they meet each other. This is especially true for the mycobiont as it is an obligately heterotrophic organism. Because of the scarcity of some photobionts as free-living in nature (Ahmadjian, 1988), especially of most species of the common photobiont genus *Trebouxia* Puym., one may ask what strategies exist to overcome this obstacle. That mycobionts may be able to capture photobionts from host lichens seems likely as some lichen taxa exhibit a facultative or obligate lichenicolous growth at their juvenile stages. Especially in the case of saturated lichen communities with delimited space resources, the development of multiple associations with two or more mycobionts and a taking over of algae by the lichenicolous taxon seem to be an important strategy (Ott, 1987*a*, *b*; Ott, Meier & Jahns, 1995). A survey of the more than 750 known potential associations of this type (including about 600 'inter-lecanoralean associations') has been presented by Rambold & Triebel (1992). The number and identity of the algal bionts in such multiple lichenized associations with two mycobionts has been proven so far only in two cases. Tschermak-Woess (1980) reported *Chaenothecopsis consociata* (Nádv.) A. Schmidt to be initially parasitic on *Chaenotheca chrysocephala* (Turner ex Ach.) Th. Fr. and being associated at that stage with the photobiont of *Chaenotheca*, *Trebouxia simplex* Tscherm.-Woess. Fully developed thalli of *Chaenothecopsis consociata*, however, have *Dictyochloropsis symbiontica* Tscherm.-Woess as

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phycobiont. The other multiple-biont association is that of *Diploschistes muscorum* (Scop.) R. Sant. and its temporary host lichen belonging to the genus *Cladonia* Hill ex P. Browne. Friedl (1987) was able to show by morphological evidence that this symbiosis of two lichens comprises two photobionts, of which the host alga, *Trebouxia irregularis* Hildreth & Ahmadjian, is replaced in the thallus of *D. muscorum* by another species, *Trebouxia showmanii* (Hildreth & Ahmadjian) Gärtner, during thallus ontogeny.

Since the process of lichenization itself cannot be observed in nature, one strategy to obtain a better understanding about the mechanisms, especially the selectivity of mycobionts in regard to the photobionts, is to examine the photobiont composition of lichen communities as a whole. It should be possible to draw generalized conclusions from the patterns of mycobiont and photobiont distribution. To obtain reliable identifications of the photobionts, their isolation and cultivation is essential. Recent studies in the taxonomy and systematics of lichen photobionts, particularly *Trebouxia* spp., focused on sequence analyses of ribosomal RNA genes. For an increasing number of algae isolated from lichens, sequence data of the small subunit (18S) rRNA gene (Friedl & Zeltner, 1994, Friedl, 1995, Bhattacharya, Friedl & Damberger, 1996), the 5′ portion of the nuclear-encoded large subunit (26S) rRNA gene (Friedl & Rokitta, 1997) and the internal transcribed spacer (ITS) rDNA (Bhattacharya *et al.*, 1996) are available. These data have been applied in the first place to investigate phylogenetic relationships among and within the groups as well as to evaluate the phylogenetic usefulness of morphological characters used in the taxonomy of coccoid green algae. In the study presented here, ITS rDNA sequence data have been applied to allow unambiguous species identification of cultivated axenic isolates from lichens and their associations or communities, respectively. As far as we know, the present study is the first where the photobiont inventory of a whole lichen community has been investigated using axenic cultures.

MATERIALS AND METHODS

Lichen samples

All algal species were taken from lichen thalli derived from sixteen test squares of the *Physcietum adscendentis* (Ochsner, 1928; Hofmann, 1993) at four sites along the river Isar north-east of München, Germany. A variant with *Phaeophyscia orbicularis* and dominating (non-lichenized) *Trentepohlia umbrina* was selected because of its frequency of occurrence in the area and a limited number of taxa to be examined. To reach the highest possible degree of sociological homogeneity of the test squares to be selected (Dierßen, 1990), the whole phorophytes were examined first. Sixteen test squares of about 20 cm^2 were fixed for detailed sociological and structural analyses. Lichen specimens collected and examined during this study are located at the lichen herbarium of the Botanische Staatssammlung München (M).

Photobionts

Photobionts were isolated by the micropipette method according to Ahmadjian (1967) and grown as described in Friedl (1989*a*). Culture strains of the isolated photobionts are maintained at the algal collections in München (M) and Kaiserslautern (KL) and are available from the authors on request. The photobionts were examined both in the lichenized and cultured state by standard light microscopic techniques. For identification, the isolated strains were compared with cultures of all known species of *Trebouxia* (Gärtner, 1985, Ettl & Gärtner, 1995) obtained from culture collections (SAG, Schlösser, 1994; UTEX, Starr & Zeikus, 1993) or were kindly provided by Dr E. Tschermak-Woess (Vienna, Austria).

DNA extraction, PCR, and sequencing

For DNA sequence analyses, five algal strains were selected from the isolated lichen photobionts (Table 1). In addition, also the ITS rDNA sequence for *Trebouxia impressa* KL-86.050E1 (isolated from *Parmelina tiliacea*) was determined. This photobiont strain is identical in ultrastructural features with the type strain of *T. impressa*, UTEX 892 (Gärtner, 1985, Friedl, 1989*b*). DNA was extracted from log phase cultures of the algae following Friedl (1996). ITS regions were amplified using the polymerase chain reaction (PCR) protocols (Saiki *et al.*, 1988) with primers and under conditions as described in Friedl (1996). The PCR products were enzymatically cleaned by the addition of 2 μl of exonuclease I and 2 μl of shrimp alkaline phosphatase (United States Biochemical, Cleveland) and kept for 15 min at 37 °C, followed by 15 min at 80 °C in a thermal cycler. After cleaning, the PCR products were sequenced directly over both strands using the dideoxy sequencing method (Sanger, Nicklen & Coulsen, 1977) with ^{35}S-labelled αdATP or with primers labelled with the fluorescent dye CY5 on an ALFexpress automatic sequencer (Pharmacia, Freiburg). Primers used for sequencing were ITS2N 5′-TCGCTGCGTTCTTCATC-3′ and ITS3N 5′-GATGAAGAACGCAGCGA-3′ which were specifically designed for ITS rDNAs of *Trebouxia* spp. and ITS 4 (White, Bruns, Lee & Taylor, 1990). The ITS rDNA sequences obtained in this study were manually aligned with all available ITS rDNA sequences for *Trebouxia* spp. (Bhattacharya *et al.*, 1996) using the multiple sequence alignment editor

Table 1. *Lichen species investigated within the* Physcietum adscendentis, *their reproductive strategy* (*v*, *vegetative reproduction ; s*, *sexual reproduction*), *the identity of their photobionts*, *the number of isolated strains and reference numbers for these strains*

Lichen species	Reproductive strategy	Photobiont	Isolated strains
Candelaria concolor	v	*T. jamesii*	1: M-96.028
Candelariella cf. *reflexa*	v	*T. jamesii*	1: M-96.011
Lecania cyrtella	s	*T. arboricola*	1: M-96.023
Lecania naegelli	s	*Dictyochloropsis symbiontica*	2: M-96.022
Lecanora spec.	s	*T. arboricola*	1: M-96.035
Lecidella elaeochroma	s	*T. arboricola*	5: M-96.013, M-96.014, M-96.019, M-96.021, M-96.032
Phaeophyscia orbicularis	v	*T. impressa*	2: M-96.009, M-96.026
Physcia adscendens	v	*T. impressa*	2: M-96.012, M-96.027
Physcia stellaris	s	*T. impressa*	1: M-96.005
Xanthoria parietina	s	*T. arboricola*	4: M-96.004, M-96.007, M-96.025, M-96.034

Underlined numbers indicate photobiont strains from which the ITS rDNAs were sequenced.

SeqEdit, written by G. J. Olsen and distributed by the Ribosomal Database Project (Maidak *et al*., 1997), and the program SeqApp (Gilbert, 1992). Our ITS rDNA sequences are available from the Genbank/EBI data base under the following accession numbers: *Trebouxia arboricola* M-96·025C1, AZ007387H; *T. arboricola* M-96·032B5, AZ007385; *T. impressa* M-96·012A2, AZ007384H; *T. impressa* M-96·026D4, AZ007386H; *T. impressa* M-96·027D1, AZ007383H; and *T. impressa* KL-86·050, AZ007388.

Data analysis

After regions of ambiguous alignment had been excluded, the alignment (Fig. 2) was subjected to two independent types of data analyses. For the distance method analysis, pairwise similarities between sequences were converted to evolutionary distances using the correction of Hasegawa, Kishino & Yano (1985) with a test version of PAUP (version 4.0d56, written by D.L Swofford and with permission of the author). Maximum parsimony analyses were performed using PAUP 4.0d56 with a random addition of sequences with 10 replicates and a branch swapping algorithm (TBR, or tree bisection–reconnection). As no suitable outgroup to the *Trebouxia* ITS rDNA sequences was available, the rDNA phylogenies were treated as unrooted. Support for internal branches in the parsimony and neighbour-joining trees were estimated using the bootstrap method (Felsenstein, 1985).

RESULTS

Ten different lichen species (Table 1) from the five test areas were recognized as members of the lichen community *Physcietum adscendentis* Ochsner. These ascomycetes species belong to the order Lecanorales, suborders Lecanorineae and Teloschistineae. The distribution of these lichen species among the test areas is shown in Table 2. Contrary to previous descriptions of the *Physcietum adscendentis*, this corticolous lichen community was also found in north-exposed to east-exposed shaded habitats close to water with relatively high atmospheric humidity and was dominated by the green alga *Trentepohlia umbrina* (Kütz.) Bornet. However, this alga was not found symbiotically within lichen thalli, but occurred only free-living and in competition with crustose and foliose lichens.

Table 2. *Inventory of lichen taxa that were present in five test areas*

Site number	1	2	3	4	5
Physcia adscendens	*	*	+		
Phaeophyscia orbicularis	+	*			
Xanthoria parietina	+	*	+		
Lecidella elaeochroma	+	*		+	+
Candelariella cf. *reflexa*	+				
Candelaria concolor	+				
Lecanora sp.		+			
Physcia stellaris			+		
Lecania naegelii				+	+
Lecania cyrtella				+	+

The test areas 4 and 5 represent pioneer areas as they were found on young trees with smooth bark. On these trees other members of the *Physcietum adscendentis* were found only in regions with rougher bark (e.g. in scars) The asterisks mark the lichens, of which the ITS rDNA of the photobionts has been determined.

Photobionts from up to five different thalli of the lichen species listed in Table 1 from the same and/or different localities were removed into axenic culture. Each mycobiont was found to be associated with only one green algal species. Each photobiont strain

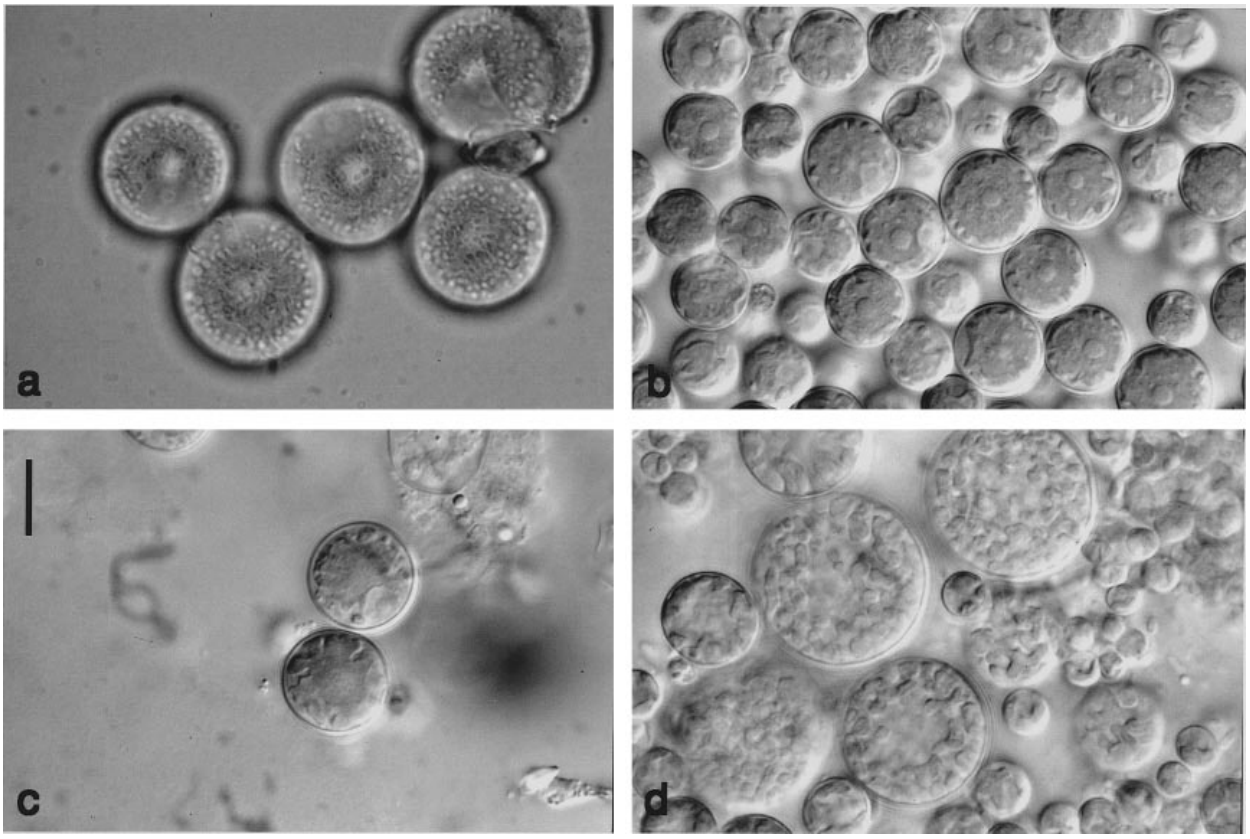

Figure 1. Chloroplast shapes of the photobionts. (*a*) Crenulate pattern of *Trebouxia arboricola*; strain M-96.013D3 from *Lecidella elaeochroma*. (*b*) Sublobate pattern of *T. impressa*; strain M-96.027D1 from *Physcia adscendens*. (*c*) Lobate pattern of *T. jamesii*; strain M-96.011X1 from *Candelariella* cf. *reflexa*. (*d*) Reticulate chloroplast in *Dictochloropsis symbiontica*, strain M-96.022B1 from *Lecania naegeli*. Scale bar, 10 μm.

listed in Table 1 represents 2–13 clones (distinguished by an additional letter and number) that originated from single cells of a lichen thallus; they were morphologically identical in all samples. Therefore, only one clone each was used for further studies. Based on light microscopical characters the photobiont strains could be assigned to three species of *Trebouxia* (*T. arboricola* Puym., *T. impressa* Ahmadjian, *T. jamesii*), and *Dictyochloropsis symbiontica*. *Trebouxia* spp. were most frequent among the investigated lichens, whereas *D. symbiontica* occurred only in *Lecania naegelii* (Hepp) Diederich & Boom. *T. arboricola* was detected in four, *T. impressa* in three, and *T. jamesii* in two lichen species (Table 1).

In strains identified as *T. arboricola* the chloroplast appeared rather finely lobed in surface view ('crenulate' pattern of Ettl & Gärtner (1984)) and the pyrenoids appeared homogenous without clearly visible structures (Fig. 1*a*). Vegetative cells of *T. impressa* had a solid chloroplast with short and broad lobes ('sublobate' pattern), the pyrenoid had a characteristic structure of parallel stripes (Fig. 1*b*). Chloroplast lobes in strains of *T. jamesii* were arranged in parallel ribs when viewed at the surface ('lobate' pattern) (Fig. 1*c*); the pyrenoids occasionally exhibited irregular incisions or stripes at their periphery and therefore resembled *T. potteri* Ahmadjian ex Gärtner and *T. gelatinosa* Ahmadjian ex Archibald, respectively, to some extent. *Dictyochloropsis* is clearly distinguished from *Trebouxia* by its parietal reticulate chloroplast and the absence of pyrenoids (Fig. 1*d*).

The ITS rDNAs from five strains that represent *T. arboricola* and *T. impressa* as identified by comparing light microscopical characters were sequenced (Tables 1, 2). Four of these strains were taken from lichen thalli of one single test square in order to investigate possible symbiotic interactions of photobionts and mycobionts within the lichen community. One clone of each photobiont strain (see above) was used for the sequence analyses. Sequence comparisons of ITS rDNAs from the isolated strains with the already available sequences and the ITS sequence from an additional strain of *T. impressa* (KL-86.050E1) generated in the present study confirmed the species identifications as based on morphological investigations. Sequences from photobiont strains identified as *T. arboricola* were highly similar to the published ITS sequences of *T. arboricola* (91·8 % similarity with strain SAG 219-1a, 99·8 % similarity with strain KL-92.011C3); the other determined photobiont sequences were 96·1–99·8 % identical with the ITS rDNA from *T.*

impressa strain KL-86.050E1 (Fig. 2). The monophyletic origin of the ITS sequences with those used for comparsions is particularly obvious by certain segments in ITS1 and ITS2 (shaded areas in Fig. 2). These segments are rather conservative in their nucleotide sequence and of specific constant length within each morphologically defined species of *Trebouxia*. Among the different species, however, they are different in length and cannot be aligned unambiguously. These highly variable segments may be used as a 'signature' sequence for each species to identify the taxonomic affiliation of an isolated strain.

For the phylogenetic analyses, 467 sequence positions could be used after regions of ambiguous alignment were excluded (Fig. 2). The data set contained 206 variant sites of which 170 were parsimony-informative characters. In the ITS rDNA phylogeny the strains identified as *T. arboricola* and *T. impressa*, respectively, form two distinct branches; they are clearly separated from lineages representing other species, *T. gelatinosa* Ahmadjian ex Archibald, *T. jamesii*, and *T. usneae* (Hildreth & Ahmadjian) Gärtner (Fig. 3). The congruence of the results from ITS rDNA sequence analyses and morphology provides a strong phylogenetic basis to use chloroplast characters for the identification of *Trebouxia* spp. In the phylogenetic analysis, *T. impressa* and *T. gelatinosa*, which have rather similar chloroplast shapes and pyrenoid morphologies, appear as closely related sister taxa. This corroborates previous findings from analyses of partial 26S rDNA sequences (Friedl & Rokitta, 1997); the congruence in both tree topologies suggests a strong phylogenetic signal in the rRNA cistron of *Trebouxia* spp. However, the ITS rDNA analyses failed to resolve other relationships among *Trebouxia* spp. A close relationship of *T. arboricola* with *T. usneae* is suggested in the neighbour-joining topology, but was only poorly supported by bootstrap analyses (Fig. 3, internal node marked by an asterisk). Maximum parsimony analyses showed *T. arboricola* as an independent lineage and suggested a closer relationship of *T. usneae* with *T. jamesii* (tree not shown), but this relationship was also insignificantly supported by bootstrap tests.

ITS rDNA analyses demonstrate the presence of genetically different strains within a species of *Trebouxia*. When comparing sequences of equal length among the strains within each species, up to 40 nucleotide positions were found to be variable except for *T. usneae*, where only a single ITS sequence is available. Among the strains of *T. impressa*, there was an average genetic distance of ITS rDNA sequences of 0·0233 (up to 19 positions different), whereas it was 0·05338 (up to 40 positions different) among strains of *T. arboricola*. Between ITS sequences of *Trebouxia* spp. already available from Genbank, the genetic differences were 0·04552 within *T. gelatinosa* (34 positions) and 0·07337 (33 positions) within *T. jamesii*. These intraspecific differences were more frequent in the highly variable sequence segments (shaded areas in Fig. 2) than in other parts of both ITS regions. The differences in ITS rDNA sequences allow a resolution of evolutionary relationships below the species level that is very important for tracing symbiotic interactions between photobionts and mycobionts of the lichen community. *T. arboricola* and *T. impressa*, respectively, fall into two lineages: the ITS rDNAs from *T. arboricola* strains that originated from *Lecidella elaeochroma* (Ach.) Choisy, *Xanthoria parietina* (L.) Th. Fr., and *Pleurosticta acetabulum* (Necker) Elix & Lumbsch were identical except for one position, but differed in 39/40 positions from the corresponding sequence of *T. arboricola* strain SAG 219-1a (Table 3, Fig. 2). In addition, the latter strain is distinguished by the presence of a 29 nts insertion in ITS2 which is lacking in other strains of the same species (Fig. 2). Strains of *T. impressa* that originated from thalli of *Physcia adscendens* (Fr.) H. Olivier from two neighbouring localities differed among each other and with a strain isolated from *Parmelina tiliacea* (Hoffm.) Hale by only three positions, but in 18/19 positions from the corresponding sequence of the same *Trebouxia* species isolated from *Phaeophyscia orbicularis* (Neck.) Moberg (Table 3, Fig. 2). Similarly, intraspecific differences are also present between strains of *T. jamesii* and *T. gelatinosa*, respectively (Table 3, Fig. 2). The differences within *T. arboricola*, *T. impressa*, and *T. jamesii* might be due to the association of these strains with taxonomically different mycobionts; they originated from lichen thalli that were from geographically close localities. The sequence differences within *T. gelatinosa*, however, may be the result of geographical separation, since both strains were from the same lichen species, *Flavoparmelia caperata* (L.) Hale, but from the USA and Germany, respectively.

DISCUSSION

The crucial step in the sexual reproductive cycle of lichenized ascomycetes is that a suitable photobiont is encountered by the germinating ascospores ('re-lichenization'). This process may be summarized in the generalized model (Fig. 4). After the contact with a potential photobiont, an inconspicuous non-stratified crust ('pre-thallus') is formed. This structure may also be formed by incompatible bionts (Ott, 1987*a*, *b*), but the stimulus that causes the transformation into a lichen thallus is provided only by compatible photobionts. The pre-thallus stage may be very important in the life cycle as it enables the mycobiont to survive until a suitable photobiont becomes available. Potential photobiont sources may be aposymbiotic algae/cyanobacteria or lichenized propagules (e.g. thallus fragments, isidia, soredia) which are from other and/or the same lichen species.

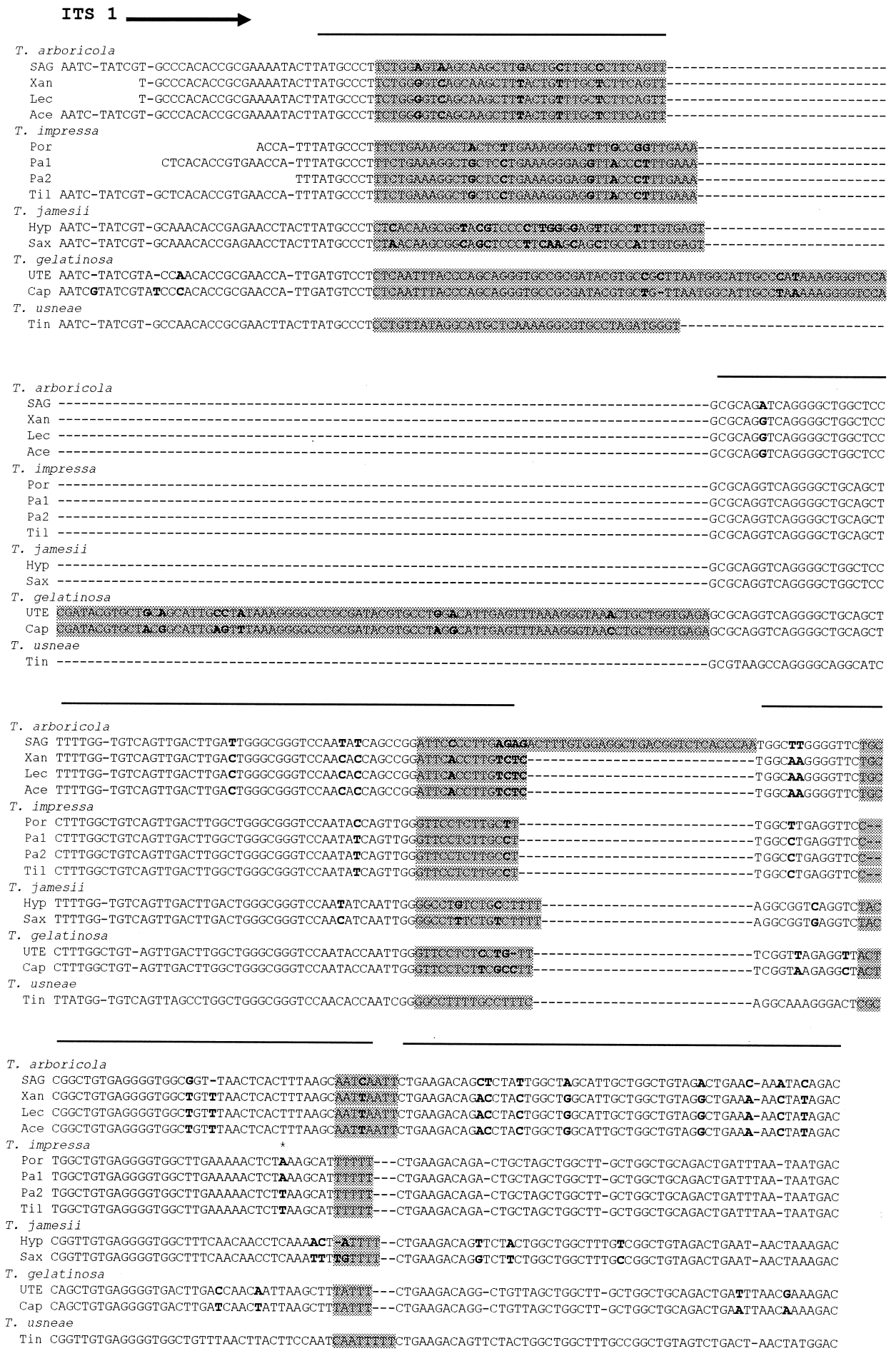

Figure 2. For legend see opposite.

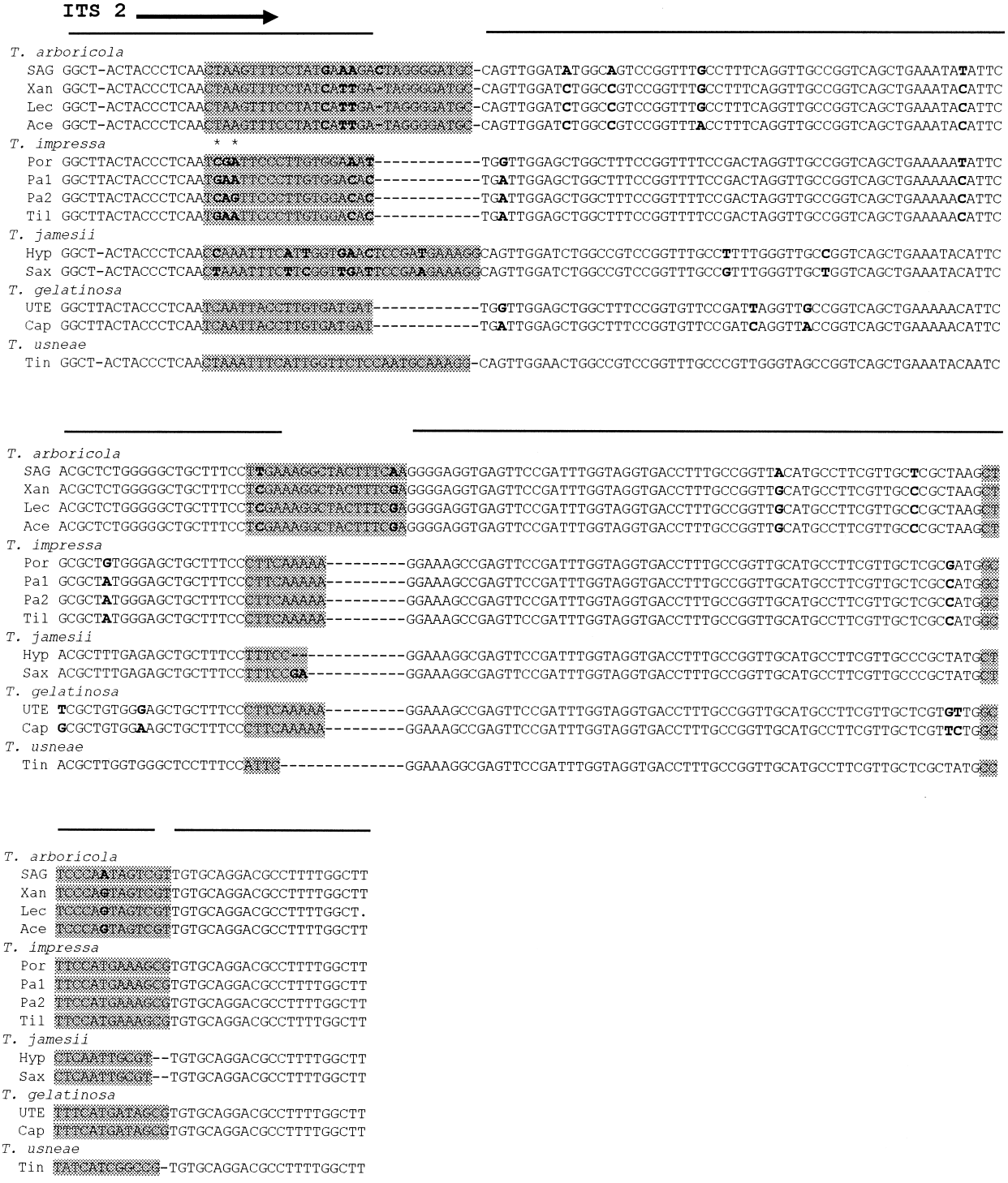

Figure 2. Alignment of the ITS rDNAs of different strains of *Trebouxia arboricola* (SAG = SAG 219-1a; Xan = M-96.025C1 from *Xanthoria parietina*; Lec = M-96.032B5 from *Lecidella elaeochroma*; Ace = KL-92.011C3 from *Pleurosticta acetabulum*), *T. impressa* (Por = M-96.026D4 from *Phaeophyscia orbicularis*; Pa1 = M-96.027D1 from *Physcia adscendens*; Pa2 = M-96.012A2 from *Physcia adscendens*; til = KL-86.050E1 from *Parmelina tiliacea*), *T. jamesii* (Hyp = KL-86.132E1 from *Hypogymnia physodes*; Sax = KL-86.156C3 from *Parmelia saxatilis*), *T. gelatinosa* (UTE = UTEX905 from *Flavoparmelia caperata*; Cap = KL-86.108B2 from *Flavoparmelia caperata*), and *T. usneae* (Tin = KL-87.019A1 from *Parmotrema tinctorum*). The line above the sequences marks regions of the alignment that were used for the phylogenetic analyses. Areas that are highly variable and different in length among the *Trebouxia* spp. are shaded (see text). Sequence differences within a *Trebouxia* species are marked in bold face letters. A dash indicates alignment gaps, a period regions for which no sequence could be obtained. Asterisks indicate sequence positions that are distinct among the two strains of *T. impressa* that have been isolated from different thalli of *Physcia adscendens* (see text).

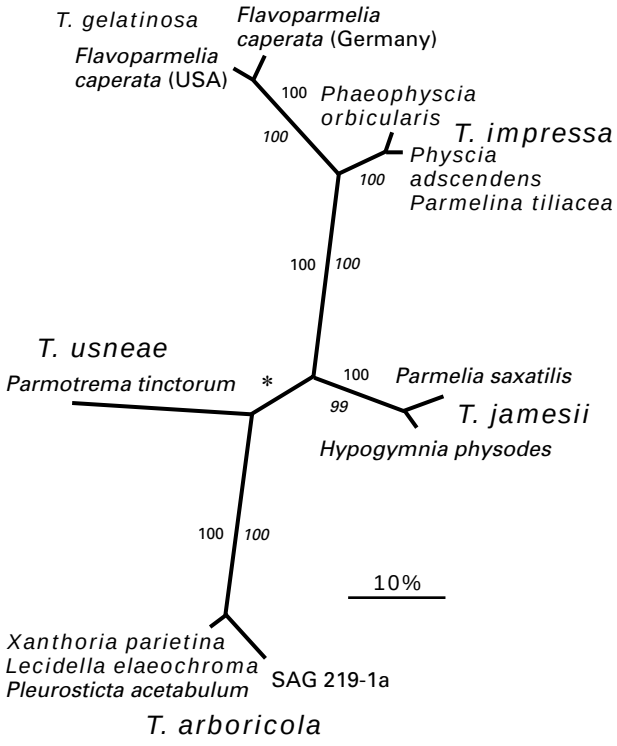

Figure 3. Unrooted phylogeny of ITS rDNA sequences from *Trebouxia* spp. using the neighbour-joining method. Bootstrap values were independently calculated for 500 replicates using neighbour-joining and unweighted maximum parsimony methods (test version of PAUP 4.0d56). An astrerisk denotes the internal branch that is not resolved by the maximum parsimony method. The distance that corresponds to 10 % sequence divergence is indicated by a scale. The ITS rDNA sequences from the photobionts of *Pleurosticta acetabulum* and *Lecidella elaeochroma*, which differed from the sequence of the photobiont from *Xanthoria parietina* in a single or no position, were not analysed but simply added to the figure. The sequences from the photobionts of *Parmelina tiliacea* and of the two samples of *Physcia adscendens* differed among each other in three or fewer positions and were also simply added to the figure.

The first recognizable sign of thallus formation is the occurrence of polarity in young primordia (Honegger, 1993). The further development into a lichen thallus may also include fusion of the initial stages. Additional compatible algal cells may be incorporated into the thallus later (Friedl, 1987; Sanders & Rico, 1992).

Two of the test squares were regarded as stands of pioneer lichens, occupying a smooth bark surface before the colonization of the other lichens (Table 2). As none of these lichens produce vegetative diaspores, the question arises from where these pioneer lichens get their photobionts. With regard to the model of re-lichenization, two sources for their photobionts seem likely. First, the algal partner may be derived from vegetative diaspores of other lichen species that have not been able to form lichen thalli in the test squares, but have disintegrated and left only their photobiont available for the re-lichenization with other mycobionts. *Pleurosticta*

Table 3. *Number of differences in sites (above diagonal, in bold face) of strains within* Trebouxia arboricola, T. impressa, T. jamesii, *and* T. gelatinosa *and pairwise corrected HKY85 pairwise distance estimates (Hashegawa et al. 1985, below diagonal) among strains of* Trebouxia *spp.*

	Tarb.SAG	Tarb.Xan	Tarb.Lec	Tarb.Ace	Timp.Por	Timp.Pa1	Timp.Pa2	Timp.Til	Tjam.Hyp	Tjam.Sax	Tgel.UTE	Tgel.Cap
Tarb.SAG	—	**39**	**39**	**40**								
Tarb.Xan	0·09102	—	**0**	**1**								
Tarb.Lec	0·09102	0·00000	—	**1**								
Tarb.Ace	0·09422	0·00220	0·00220	—								
Timp.Por	0·58163	0·60231	0·6031	0·60199	—	**18**	**19**	**18**				
Timp.Pa1	0·62537	0·61783	0·61783	0·61753	0·04052	—	**3**	**1**				
Timp.Pa2	0·63170	0·62375	0·62375	0·62342	0·04319	0·00664	—	**2**				
Timp.Til	0·61686	0·60943	0·60943	0·60912	0·04303	0·00219	0·00441	—				
Tjam.Hyp	0·47273	0·42118	0·42118	0·42932	0·45829	0·43560	0·43850	0·43706	—	**33**		
Tjam.Sax	0·51281	0·44059	0·44059	0·44914	0·51541	0·48612	0·49970	0·48226	0·07337	—		
Tgel.UTE	0·78477	0·77684	0·77684	0·77642	0.24145	0·27636	0·27227	0·27160	0·61993	0·53583	—	**34**
Tgel.Cap	0·78485	0·75393	0·75393	0·75347	0·26227	0·27747	0·27342	0·27270	0·62218	0·54629	0·04552	—
Tusn.Tin	0·50053	0·39873	0·39873	0.40620	0·72464	0·69006	0·69654	0·68060	0·34721	0·37431	0·74622	0·71595

Numbers in boxes show differences in sites and genetic distances within a species of *Trebouxia*. Tarb.SAG, *T. arboricola* SAG 219-1a; Tarb.Xan, M-96.025C1 from *Xanthoria parientina;* Tarb.Lec, *T. arboricola* M-96.032B5 from *Lecidella elaeochroma;* Tarb.Ace, *T. arboricola* KL-92.011C3 from *Pleurostica acetabulum;* Timp.Por, *T. impressa* M-96.026D4 from *Phaeophyscia orbicularis;* Timp.Pa1, *T. impressa* M-96.027D1 from *Physcia adscendens;* Timp.Pa2, M-96.012A2 from *Physcia adscendens;* Timp.Til, *T. impressa* KL-86.050E1 from *Parmelina tiliacea;* Tjam.Hyp, *T. jamesii* KL-86.132E1 from *Hypogymnia physodes;* Tjam.Sax *T. jamesii* KL-86.156C3 from *Parmelia saxatilis;* Tgel.UTE, *T. gelatinosa* UTEX905 from *Flavoparmelia caperata;* Tgel.Cap, *T. gelatinosa* KL-86.108B2 from *Flavoparmelia caperata;* Tusn.Tin, *T. usneae* KL-87.019A1 from *Parmotrema tinctorum.*

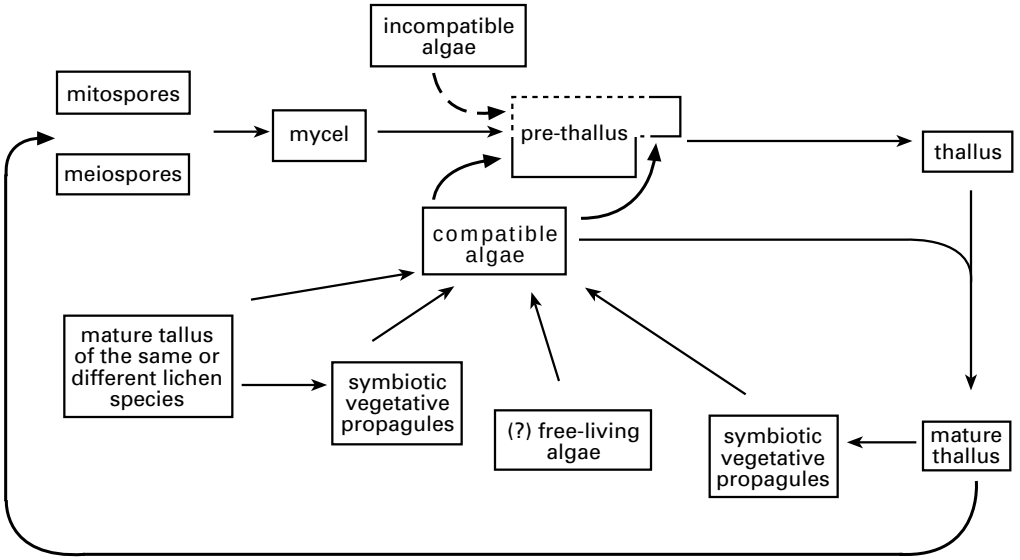

Figure 4. An updated model of re-lichenization in the reproductive cycle of lichenized ascomycetes. The germinating fungal spore (mito- or meiospore) forms a mycelium. After contact with a potential photobiont (first bold line), an inconspicuous non-stratified crust ('pre-thallus') is formed. This structure can also be formed with incompatible algae (broken bold line), but for further development the compatible photobiont is essential (second bold line), because the stimulus that causes the transformation into a lichen thallus is provided only by compatible photobionts. The pre-thallus stage might be very important in the life cycle as it enables the mycobiont to survive until a suitable photobiont becomes available. Potential photobiont sources can be aposymbiotic algae/cyanobacteria or lichenized propagules (e.g. thallus fragments, isidia, soredia) which are from other and/or the same lichen species. Additional compatible algal cells might be incorporated into the thallus later. With the production of spores the life cycle is closed.

acetabulum, whose photobiont has been found to be morphologically identical with that of the pioneer lichens, might be an example of such a lichen that could not inhabit the test squares, but might provide photobionts via its vegetative diaspores. However, this possibility seems unlikely as no thallus of *Pleurosticta acetabulum* was found within a reasonable distance for the vegetative diaspores to reach the investigated trees. The other possibility is the existence of free-living populations of *Trebouxia arboricola*, large enough to favour re-lichenization at a reasonable frequency. Also *Lecania naegelii* may be regarded as a pioneer lichen in our test squares. As its photobiont, *Dictyochloropsis symbiontica*, is also known to occur in the non-lichenized state (Tschermak-Woess, 1980; Handa & Nakano, 1988), it seems rather plausible that germinating ascospores of *L. naegelii* might have encountered free-living cells of *D. symbiontica*.

Although cells of *Trebouxia* have been observed not associated with lichen fungi in nature (Degelius, 1964; Tschermak-Woess, 1978; Bubrick, Galun & Frensdorff, 1984; Mukhtar, Garty & Galun, 1994), it is not known whether these free-living algal cells have escaped from lichen thalli/propagules just for a short period of time or have developed independently of lichen fungi (Ahmadjian, 1988). The rare reports on *Trebouxia* found outside of lichen thalli might indicate that *Trebouxia* is at least not a common member of the free-living algal community. Support for the presence of non-lichenized *Trebouxia* colonies might come from our observation that *Lecania cyrtella*, *Lecidella elaeochroma*, and *Lecanora* spec. were found as pioneer lichens on the bare and smooth bark of young ash trees. These lichens reproduced exclusively by ascospores while they had the same photobiont species, *T. arboricola*. Since the smooth bark surface of such young trees is not a suitable substratum for the adherence and further development of lichenized propagules as the water relations of these substrata might be unfavourable (Barkman, 1958; Schuster, Ott & Jahns, 1985), it seems very unlikely that this alga has been provided by symbiotic diaspores or thallus fragments of other lichens. Therefore, we assume that non-lichenized cells of *Trebouxia arboricola* formed the photobiont source for the pioneer lichens in the test squares, rather similar to the situation of *Lecania naegelii* where *Dictyochloropsis symbiontica* may have been taken as symbiont from the free-living state. It is important to note that the free-living populations of *T. arboricola* need not be very large, supposing that the pre-thallus is a common feature in lichens. Such a stage would enable the mycobiont to survive some time without a compatible photobiont. Gärtner (1985) mentioned a possible free-living origin for *T. arboricola* strain SAG 219-1a. Although the strain of *T. arboricola* from the lichens studied here is not identical in their ITS rDNA sequence to the sequence from strain SAG 219-1a, there is no reason not to regard both strains as conspecific since their morphology is identical and their monophyletic origin is strongly supported by the ITS rDNA sequence analyses.

Following the model of re-lichenization, it seems plausible that the lichenized propagules of the sterile lichens *Physcia adscendens* and *Phaeophyscia orbicularis*, which were abundant in the test squares, serve as photobiont sources for germinating ascospores of *Xanthoria parietina* that does not produce vegetative diaspores. Based on field observations, Ott (1987*a*, *b*) suggested that germinating ascospores of *X. parietina* might take over the photobiont from soredia of *Physcia* species. However, the morphological and molecular characterization of photobionts from our test squares show that *X. parietina* and *Physcia adscendens*/*Phaeophyscia orbicularis* contain two distinct species of photobionts. The analyses of ITS rDNA sequences show that the evolutionary distance between the photobionts from *X. parietina*, *T. arboricola*, and from *P. adscendens*/*P. orbicularis*, *T. impressa*, is too long to regard them as conspecific. The distance between the ITS sequences from photobionts of *P. adscendens* and *X. parietina* (0·61783/0·62375) is about twelve times larger than the average distance found within *Trebouxia* species (0·04890). This finding excludes the possibility that the lichenized propagules of *P. adscendens* and *P. orbicularis* may have acted as a photobiont source for *X. parietina* ascospores, at least for the lichen samples studied here. The finding that *Xanthoria parietina* and *Physcia* spp. have different photobionts is also supported by previous studies, although their results have to be taken with caution as species identification appears to be quite difficult when not based on extensive comparisons with all other available strains (Gärtner, 1985; Friedl, 1989*b*). According to earlier studies many foliose lichen species of the Physciaceae can have *Trebouxia impressa* or *Trebouxia flava* Archibald (the latter being possibly very closely related or even conspecific with *T. impressa*, Friedl, 1989*b*), e.g. *Physcia stellaris* (L.) Nyl. (Ahmadjian, 1960), *Physconia distorta* (With.) J. R. Laundon (as *Physconia pulverulenta*, Archibald, 1975), *Heterodermia comosa* (Eschw.) Follmann & Redon and *H. dendricata* (Pers.) Poelt (Wang-Yang, 1968). However, these *Trebouxia* species are not known as photobionts of *Xanthoria parietina*. For *X. parietina*, so far only *T. aggregata* (Archibald) Gärtner, which might be conspecific with *T. arboricola* (see discussion in Friedl, 1989*b*), *T. arboricola*, and *T. italiana* Archibald have been reported to be photobionts (Archibald, 1975, Gärtner, 1985, Honegger, 1996).

ITS sequence analyses rather suggest that *Xanthoria parietina* and *Lecidella elaeochroma* share the same 'photobiont-pool' as their algal partners were found to be identical in their ITS rDNA sequence (with only one sequence position difference). Small thallus pieces of other lichens in our test squares that contained *Trebouxia arboricola*, e.g. *Lecidella elaeochroma*, *Lecania cyrtella*, *Lecanora* sp. may have made their photobionts available to *X. parietina*. Although these lichens did not form lichenized propagules, grazing snails might have enabled a photobiont transfer. Another possibility is that *X. parietina* might also act as a lichenicolous lichen and takes over the photobiont directly from a host lichen, since *X. parietina* was found growing directly on thalli of *Lecidella elaeochroma* in the test squares. Further, photobionts from disintegrating thallus pieces of other lichens that were unable to further develop within the test squares (e.g. *Pleurosticta acetabulum*) might have been captured by *X. parietina*. Finally, free living cells of *T. arboricola* might have served as the photobiont source. All photobiont sources indicated in Figure 4 are likely to occur, and, as based on our data, none can be regarded to be predominant.

Photobionts can be frequently exchanged owing to the obligate re-lichenization process in sexually reproducing lichens. However, in lichens that reproduce vegetatively there might be no exchange of photobionts and, therefore, the photobionts of different lichen thalli may not constitute a genetically homogenous pool. This hypothesis may explain why the *T. impressa* strains that are the photobionts from *Phaeophyscia orbicularis* and *Physcia adscendens*, are genetically different. Both lichen species were sterile and exclusively reproduced by lichenized propagules in the test squares. The intraspecific distinctness between strains of *T. jamesii* isolated from the sterile lichens *Parmelia saxatilis* (L.) Ach. (only occasionally found with apothecia) and *Hypogymnia physodes* (L.) Nyl. (found at different localities in Germany) may further support this hypothesis. The exchange of photobionts, however, might also be possible in sterile lichens as is suggested by the high genetic identity of the photobionts from *Physcia adscendens* and *Parmelina tiliacea* (only one or two sequence positions different). Symbiotic propagules from other lichens with a different photobiont might become incorporated (e.g. at early stages of the thallus development) and the accessory alga later might become dominant within the growing thallus. That the photobiont may be exchanged during the development of lichen thalli has already been observed in *Diploschistes muscorum* (Friedl, 1987).

Among the photobionts investigated in this study, *Dictyochloropsis symbiontica* and *Trebouxia arboricola* are obviously restricted to lichens which do not develop lichenized propagules such as soredia, isidia, pseudocyphellae, etc. However, there are reliable records for *T. arboricola*, *T. impressa* and *T. jamesii* that these species occur as photobionts of both lichens, those that reproduce by asexually formed symbiotic diaspores and those reproducing sexually without symbiotic diaspores. Reports (Ahmadjian, 1993) indicate that, aside from the reports given here, the three species of *Trebouxia* that were investigated in this study are common also in lichens

of other suborders of the Lecanorales: *T. arboricola* also occurs in the Stereocaulaceae, Lecideaceae, Parmeliaceae, Harpidiaceae and Teloschistaceae; *T. impressa* is also present in the Parmeliaceae, Physciaceae and Cladoniaceae; *T. jamesii* is also known from Anziaceae, Parmeliaceae and Schaereriaceae. *T. arboricola* has been found in lichens reproducing asexually, e.g., *Flavopunctelia flaventior* (Stirton) Hale, *Melanelia exasperatula* (Nyl.) Essl. and *Parmelia saxatilis* (Friedl, 1989*b*). *T. impressa* occurs for example in the sexually reproducing *Physcia stellaris* (Ahmadjian, 1960, this study) and *Parmelina quercina* var. *carporrhizans* (Taylor) Poelt & Vezda (Friedl, 1989*b*), but also in the mostly asexual lichens *Phaeophyscia orbicularis* and *Physcia adscendens* (this study). *T. jamesii* has been reported from the sexually reproducing lichens *Anzia colpodes* (Ach.) Stizenb. and *Imshaugia placorodia* (Ach.) S. L. F. Meyer (Friedl, 1989*b*), but also from the mostly sterile lichens *Hypogymnia physodes* and *Parmelia saxatilis* (Friedl, 1989*b*). Therefore the occurence of a certain photobiont species seems not to be correlated with a certain type of reproduction of the lichen. To estimate the preferential interaction with a defined mycobiont taxon, i.e. selectivity *sensu* Galun & Bubrik (1984), additional photobiont records, preferably supported by molecular data, are necessary. To clarify the conditions of selectivity of photobiont choice, examinations on lichen photobionts will have to be extended to different lichen communities and geographical regions.

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