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PHOTOBIONT INVENTORY OF A LICHEN COMMUNITY GROWING ON HEAVY-METAL-RICH ROCK

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Abstract: The photobiont inventory of a stand of the *Acarosporetum sinopicae*, a lichen community comprising saxicolous, chalcophilous lichens, has been analysed. Investigated lichen species were *Acarospora rugulosa*, *A. sinopica*, *Bellemerea diamartha, Lecanora polytropa, L. subaurea, Lecidea silacea, L. lapicida, Rhizocarpon geographicum*, and *Umbilicaria cylindrica*. For all these lichen species this is the first record of the photobionts, except for *L. lapicida*. The photobionts were cultured axenically and investigated using light microscopical and molecular methods (ITS-sequence analyses). Every lichen species contained only one photobiont species. All photobionts belong to *Trebouxia jamesii*, but two different subspecies were found with the morphological differences corresponding to molecular differences. The new subspecies *T. jamesii* subsp. *angustilobata* is described, differing from the typical *T. jamesii* by a crenulate chloroplast but identical to the latter taxon in respect to the pyrenoid structure in the light microscope. These results are discussed with respect to the photobiont inventory of the *Physcietum adscendentis*, analysed in an earlier study.

Introduction

Lichen fungi reproducing by spores need to find compatible algae in order to form a new lichen thallus (re-lichenization). Sexually reproducing lichens can grow only where suitable photobionts are present and thus the available photobionts determine to some extent the composition of lichen communities. A possible source of photobionts may be free-living populations of algae (the existence of these is controversial) or parts of thalli of other lichens (e.g. vegetative diaspores, thallus fragments) that contain the compatible photobiont. To investigate possible sources of photobionts, it is necessary to know the algal partners of all lichen species occurring in the same community, because the acquisition of photobionts is only meaningful when lichen fungi form lichens with the same algal species. These relationships may vary between different communities and, therefore, it is important to know the composition of the photobiont pool in the lichen communities. If the same lichen species occurs in two different communities, it is possible to investigate the selectivity of photobiont choice of the same lichen species under varying ecological conditions (e.g. climate, substratum, competition, available photobionts, etc.). Also a possible co-evolution (a reciprocal influence on the

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evolution of interacting species) between the bionts involved can be traced more easily, as information about the photobionts in co-occurring lichens may be important in the evaluation of this process. Only when the whole photobiont spectrum is known, can one decide whether photobiont choice was determined mainly by the available photobionts or due to high fungal selectivity, possibly by co-evolution.

If the communities do not comprise common members, it will be interesting to compare the photobiont inventory of different types of communities such as those growing on different types of substratum (e.g. bark, soil, rock, etc.), pioneer versus sociologically established stands, communities comprising different lichen growth types, or communities consisting of taxonomically related or unrelated members. Furthermore the data obtained in these studies are also very useful for testing the recently proposed role of photobionts as possible indicators of phylogenetic relationships in lichens (Rambold *et al.* 1998). For these reasons it is desirable to analyse geographically separated stands of one and the same community and to extend the investigations of the photobiont inventory to a terricolous community.

Recently we investigated the photobiont inventory of a *Trentepohlia umbrina* (Kütz.) Bornet dominated variant of the *Physcietum adscendentis* Ochsner (Beck *et al.* 1998). In order to increase our knowledge about the photobiont inventory of lichen communities, in this study one stand of the *Acarosporetum sinopicae* Hilitzer, which comprizes saxicolous, chalcophilous lichens (e.g. growing on heavy-metal-containing rock) has been analysed. The photobionts were cultured axenically and determined using light microscopical and molecular data [internal transcribed spacer (ITS) rDNA sequencing].

Materials and Methods

Lichen samples

All algal strains examined were isolated from lichen thalli derived from a single rock, containing iron sulphate, at the 'Schwarze Wand' near Hüttschlag, Salzburger Land, Austria (1700 m alt.). The lichen species belonged to the community *Acarosporetum sinopicae* (e. g. Wirth 1972; Purvis & Halls 1996). Lichen specimens collected and examined during this study are deposited at the Botanische Staatssammlung München (M).

Photobionts

Photobionts were isolated by the micro-pipette method according to Ahmadjian (1967) and grown as described in Friedl (1989). Culture strains of the isolated photobionts are maintained at the Institut für Systematische Botanik, LMU München. 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, one clone of each algal strain was selected from the isolated lichen photobionts (see Table 1). This was considered to be sufficient, as all clones from one strain were found to be morphologically identical. DNA was extracted from log-phase cultures of the algae following Friedl (1996). ITS regions were amplified using the polymerase chain reaction (PCR) protocol (Saiki *et al.* 1988) with primers and under conditions as described in Friedl (1996). The

TABLE 1. *Isolated algal strains, number of clones obtained and investigated by light microscopy and the clones used for sequencing*

Lichen species	Reproductive strategy*	Photobiont (*T. jamesii* subsp.)	Number of clones investigated	Sequenced clone
Acarospora rugulosa	S	*jamesii*	10	M-97·019C3
Bellemerea diamartha	S	*jamesii*	3	M-97·020B4
Lecanora polytropa	S	*jamesii*	4	M-97·022B3
L. subaurea	V	*jamesii*	6	M-97·025B5
Lecidea silacea	S	*jamesii*	3	M-97·017A2
Rhizocarpon geographicum	S	*jamesii*	5	M-97·021C5
Umbilicaria cylindrica	S	*jamesii*	5	M-97·024C2
Acarospora sinopica	S	*angustilobata*	2	M-97·026C4
Lecidea lapicida	S	*angustilobata*	6	M-97·027B3

*S=sexual; V=vegetative

PCR products were cleaned by Qiaquick (Qiagen) spin columns, following instructions in the manual and sequenced directly over both strands using a Licor X4 automated sequencer. Sequencing primers were 1800F (Friedl 1996) and ITS 4 (White *et al.* 1990). The ITS rDNA sequences obtained in this study were manually aligned with all available ITS rDNA sequences for *Trebouxia* species (Bhattacharya *et al.* 1996; Beck *et al.* 1998). The new ITS rDNA sequences are available from the Genbank/EBI data base under the following accession numbers: *Trebouxia jamesii* M-97·017A2: AF128270 and *T. jamesii* subsp. *angustilobata* M-97·027B3: AF128271.

Data analysis

After regions of ambiguous alignment had been excluded (in ITS1: 44–187, 265–298, 335–359 and in ITS2: 26–47, 127–144; positions are given in respect to the alignment shown in Beck *et al.* 1998), the alignment with 383 nucleotides was subjected to maximum parsimony analyses using PAUP 3·1·1 (Swofford 1993). The analyses were done using a branch-and-bound search and the resulting phylogram was midpoint-rooted, as no suitable outgroup for *Trebouxia* species was available. Stability of monophyletic groups was tested using the bootstrap method (Felsenstein 1985) with 500 replications.

Results

At the site studied, nine different lichen species (Table 1) were analysed with respect to their photobionts. All species belong to the ascomycete order Lecanorales and have been previously reported to occur on heavy-metal-containing substrata, being members of the *Acarosporetum sinopicae* (e.g. Wirth 1972; Creveld 1981; Purvis & Halls 1996). Only two taxa of photobiont were found in the community investigated, both belonging to *T. jamesii* (Hildreth & Ahmadjian) Gärtner. A new subspecies of *T. jamesii* is described based on differences in the chloroplast morphology (see Table 2 and Fig. 1) and ITS sequence.

Between two and ten photobiont clones were obtained from each lichen thallus (listed in Table 1). Each mycobiont was found to be associated with only one photobiont species, as all of the clones obtained from the same lichen thallus were identical with respect to morphological characters. Based on light

TABLE 2. *Comparison of morphological characters from* Trebouxia jamesii *and* T. arboricola

Character	*T. jamesii* subsp. *jamesii*	*T. jamesii* subsp. *angustilobata*	*T. arboricola*
Chloroplast shape*			
Lobes	Broad, twisted	± Small, regular	Small, regular
Surface view	Rips to big patches	Small patches (± 'crenulate')	'Crenulate'
Pyrenoid	Indistinct	Indistinct	Well visible ± angular
Cell size [µm]	(4 −)6–20	(4 −)6–20	(4 −)6–23

*Definitions of chloroplast shapes follows Gärtner (1985).

microscopical features, most of the photobiont strains (Table 1) could be assigned to *T. jamesii* subsp. *jamesii*, the pyrenoid structure and chloroplast morphology fitting the description of this species. Such strains were obtained from *Bellemerea diamartha, Lecanora subaurea, Lecidea silacea* (known only from heavy-metal-containing rock) and *Acarospora rugulosa, Lecanora polytropa, Rhizocarpon geographicum, Umbilicaria cylindrica* (also known from siliceous rock without heavy metals). However, two strains (Table 1), here called *T. jamesii* subsp. *angustilobata*, were found to have a similar pyrenoid structure, with light microscopy, but different chloroplast morphology. The chloroplast lobes of these two strains are much finer than the chloroplast lobes of the other strains (belonging to *T. jamesii* subsp. *jamesii*), and therefore resemble the fine chloroplast lobes of *T. arboricola* (for comparison of these three taxa see Table 2 and Fig. 1). These strains were obtained from *A. sinopica* and *Lecidea lapicida*. Strictly following the determination keys to *Trebouxia* species (e.g. Gärtner 1985; Ettl & Gärtner 1995) these strains would key out as *T. arboricola* Puym./*T. crenulata* Archibald/*T. incrustata* Ahmadjian ex Gärtner, but did not really fit the description of these species. As this combination of diagnostic characters (a crenulate chloroplast and a pyrenoid, which is identical to that of *T. jamesii* under the light microscope) has not been reported before, the taxon *T. jamesii* subsp. *angustilobata* is described below, as a new subspecies.

Examination of the ITS rDNA region confirmed the morphological observations. All strains with similar morphology were identical in their ITS sequence, except for a clone obtained from *Bellemerea diamartha* with two base substitutions compared to the others. The number of base changes between strains of *T. jamesii* subsp. *jamesii* and *T. jamesii* subsp. *angustilobata* were 25 (6·0%) in ITS1 and 21 (8·6%) in ITS2. In order to test for the phylogenetic position of *T. jamesii* subsp. *angustilobata*, the sequences obtained were aligned with all other published *Trebouxia* ITS sequences and an unrooted phylogeny was constructed. The cladistic analysis produced one most parsimonious tree (Fig. 2) with a consistency index (CI) of 0·805. The resulting phylogram clearly indicates that the *T. jamesii* strains obtained, with a similar pyrenoid structure (subsp. *jamesii* and subsp. *angustilobata*), are closely related (with a bootstrap support of 95). This result is confirmed by the low pairwise mean

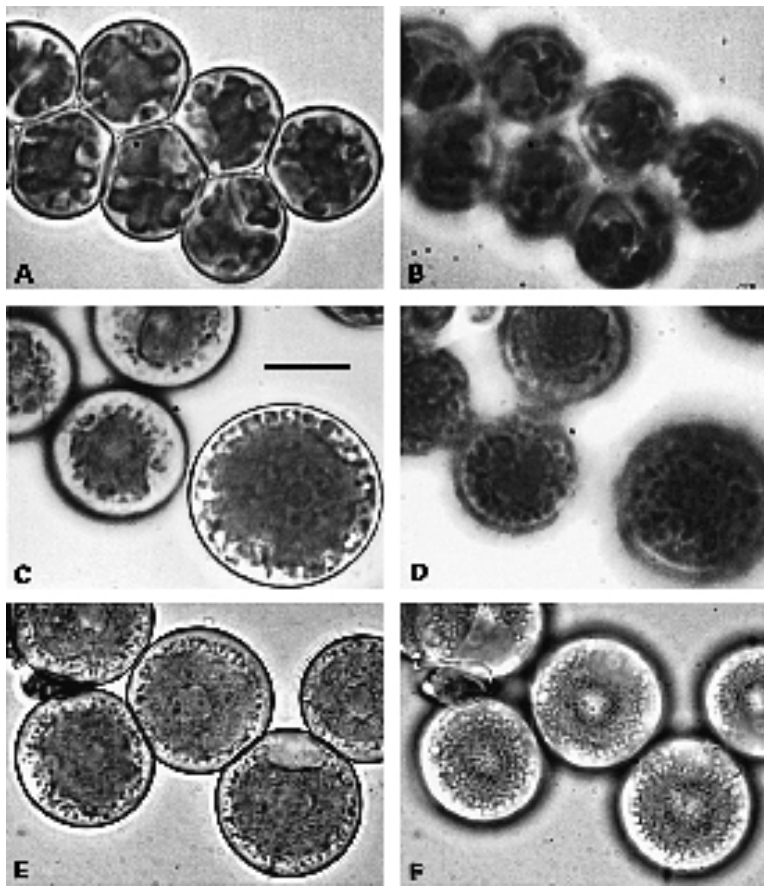

FIG. 1. Chloroplast shapes of the different subspecies of *Trebouxia jamesii* (A, B, subsp. *jamesii*; C, D, subsp. *angustilobata*) and of *T. arboricola* (E, F). A, C, E in optical section and B, D, F in surface view.

distance (Table 3) between these taxa (around 0·05) and high values between these strains and *T. arboricola* (around 0·15). These findings unequivocally demonstrate that *T. jamesii* subsp. *angustilobata* belongs to *T. jamesii*, although having a very similar chloroplast morphology to *T. arboricola*. The low bootstrap values within the grouping of the strains of *T. jamesii* indicate that the exact relationship between these strains is not yet clear. These findings underline that the pyrenoid structure offers important information in the identification and classification of *Trebouxia* species.

The occurrence of one of the two subspecies of *T. jamesii* and the taxonomic position of the mycobiont, the mode of reproduction, the colour and growth form of the lichen thallus were found not to be correlated with each other.

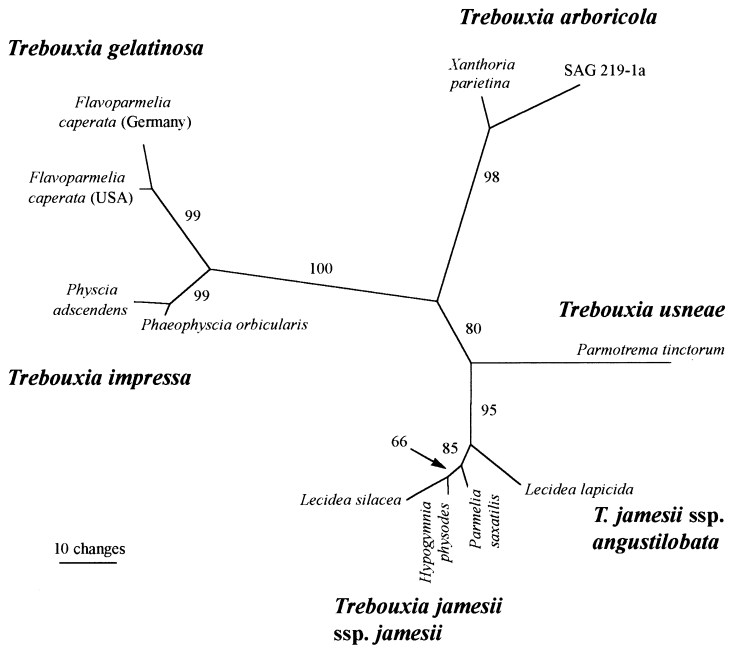

FIG. 2. Unrooted phylogeny of *Trebouxia* ITS rDNA sequences using the maximum parsimony method and a branch-and-bound search (383 aligned nucleotides; see Materials and Methods). Bootstrap values were independently calculated for 500 replicates using unweighted maximum parsimony methods. The scale indicates the distance due to ten evolutionary steps.

Discussion

The photobiont inventory of the *Acarosporetum sinopicae*, as far as analysed, is much poorer than the one of the *Physcietum adscendentis* (Beck *et al.* 1998), although a similar number of lichen species have been examined (9 and 10 species respectively). In the *Physcietum adscendentis* five photobiont species were found (*Trebouxia arboricola*, *T. gelatinosa*⋆ Ahmadjian ex Archibald, *T. impressa* Ahmadjian, *T. potteri*⋆ Ahmadjian ex Gärtner and *Dictyochloropsis symbiontica* Tscherm.-Woess; the species marked with an asterix were reported to belong to *T. jamesii*); at least two at the same stand. Like the mycobionts from the *Acarosporetum sinopicae* analysed in this study, those from the *Physcietum adscendentis* were selective towards their photobiont as only one algal species was found in one lichen species. However, the selectivity of the mycobionts from the *Acarosporetum sinopicae* is blurred, because only one photobiont species has been found so far. Nevertheless preliminary results from other localities indicate that these mycobiont species are indeed selective. Owing to the greater variety of photobionts in the *Physcietum adscendentis*, selectivity of the mycobionts in that community was supported by the observation that lichen species growing closely together may have different photobionts. Indications for co-evolution mechanisms between a mycobiont and a photobiont species were not found in the *Acarosporetum sinopicae*

TABLE 3. *Pairwise mean distances between strains of* Trebouxia*

	Tarb.SAG	Tarb.Xpar	Timp.Porb	Timp.Pads	Tjj.Hphy	Tjj.Psax	Tjj.Lsil	Tja.Llap	Tgel.UTE	Tgel.Fcap	Tusn.Ptin
Tarb.SAG	–										
Tarb.Xpar	0·058	–									
Timp.Porb	0·198	0·193	–								
Timp.Pads	0·201	0·193	0·021	–							
Tjj.Hphy	0·162	0·143	0·183	0·181	–						
Tjj.Psax	0·164	0·141	0·186	0·181	0·027	–					
Tjj.Lsil	0·162	0·143	0·183	0·178	0·024	0·034	–				
Tja.Llap	0·156	0·146	0·191	0·186	0·050	0·048	0·053	–			
Tgel.UTE	0·237	0·232	0·077	0·088	0·198	0·196	0·198	0·212	–		
Tgel.Fcap	0·243	0·232	0·096	0·099	0·206	0·204	0·206	0·223	0·029	–	
Tusn.Ptin	0·198	0·164	0·228	0·228	0·149	0·143	0·149	0·154	0·241	0·243	–

*Numbers show genetic distances within strains of *Trebouxia*. Tarb SAG, *T. arboricola* SAG 219-1a; Tarb.Xpar, *T. arboricola* M-96·025C1 from *Xanthoria parientina*; Timp.Porb, *T. impressa* M-96·026D4 from *Phaeophyscia orbicularis*; Timp.Pads, *T. impressa* M-96·027D1 from *Physcia adscendens*; Tjj.Hphy, *T. jamesii* subsp. *jamesii* KL-86·132E1 from *Hypogymnia physodes*; Tjj.Psax, *T. jamesii* subsp. *jamesii* KL-86·156C3 from *Parmelia saxatilis*; Tjj.Lsil, *T. jamesii* subsp. *jamesii* M-97·017A2 from *Lecidea silacea*; Tja.Llap, *T. jamesii* subsp. *angustilobata* M-97·027B3 from *Lecidea lapicida*; Tgel.UTE, *T. gelatinosa* UTEX 905 from *Flavoparmelia caperata*; Tgel.Fcap, *T. gelatinosa* KL-86·108B2 from *Flavoparmelia caperata*; Tusn.Ptin, *T. usneae* KL-87·019A1 from *Paramotrema tinctorum*.

whereas in the *Physcietum adscendentis* the observed phenomenon of co-development may have resulted in co-evolution. For instance, *Trebouxia impressa*, isolated from thalli of *Physcia adscendens*, collected from different localities, were more closely related to each other in respect to their ITS sequence than to the *T. impressa* clone obtained from *Phaeophyscia orbicularis*, collected in one of these localities as well. This indicates that the photobionts of these two lichens, reproducing mainly by lichenized propagules, were separated and therefore could evolve separately. But an exchange of photobionts seems to be possible, even in lichens reproducing vegetatively (Beck *et al*. 1998).

The ecophysiologically extreme environment of these lichens can be considered as an explanation for the occurrence of only one photobiont species in the *Acarosporetum sinopicae*. Only well-adapted photobiont clones may be able to grow there. A first hint in this direction can be drawn from the observation that the isolated clone *T. jamesii* subsp. *jamesii* (M-97·017A2) is able to grow even in liquid solutions with 70 mM iron ($FeCl_2$), whereas the type strain of *T. jamesii* (UTEX 2233), isolated from *Schaereria fuscocinerea* (Nyl.) Clauzade & Cl. Roux (=*S. tenebrosa* (Flot.) Hertel & Poelt, not growing on heavy-metal-containing substrata) is not capable of tolerating such high iron concentrations (Beck, unpublished). On the other hand, it has to be considered that not all members of the *Acarosporetum sinopicae* have been investigated yet and the rather low number of lichens with vegetative diaspores (only one in comparison to five in the *Physcietum adscendentis*) may be another explanation for the relatively poor photobiont inventory. Since only vegetatively reproducing lichens are able to transport new (not free-living) photobiont species into the stand, the number of photobionts in a community might be correlated with the number of vegetatively reproducing lichens present. A correlation of that kind would also be in accordance with the rarity of *Trebouxia* cells outside lichen thalli. The investigation of further stands of the *Acarosporetum sinopicae* will help to clarify whether a relationship between the number of photobionts and the number of vegetatively reproducing lichens exists in this community.

This discussion reveals that the photobiont inventory of lichen communities is not influenced by only one factor. To the contrary, several factors, such as selectivity of the mycobionts or ecological demands of the photobionts, have to be considered. Whether the photobiont strains with different morphologies represent closely related but different species rather than different subspecies is difficult to decide at the moment. They show rather big differences in their ITS sequence (46 exchanged bases) in respect to known differences of clones from *T. impressa* (19), but the number is only slightly higher than in the ITS sequences of clones from *T. arboricola* (39; Beck *et al*. 1998). Therefore, it seems preferable not to start creating new species of *Trebouxia* until more is known about genetic and morphological variability (correlated to the genetic ones) in this genus. For this reason, the clones of *T. jamesii* obtained in this study are treated as different subspecies, based on the same pyrenoid structure but morphologically different chloroplast shapes, corresponding to differences in the ITS region. The characteristics of this new subspecies are as follows:

Trebouxia jamesii (Hildreth & Ahmadjian) Gärtner subsp. angustilobata A. Beck subsp. nov.

Cellulis globosis, diametro usque ad 20 µm. Differt a subspecie *jamesii* chromatophoro subtiliter lobato.

Typus: M-97·027B3, (dried culture on agar, M—holotype; living cultures, SAG); isolated from *Lecidea lapicida* (Ach.) Ach., Austria, Salzburger Land, 'Schwarze Wand' near Hüttschlag, 30 viii 1997, A. Beck 30 (M, sub *L. silacea*).

Fig. 1C & D

Cells spherical, (4–)6–20 µm diam., with a thin cell wall. The axial chloroplast is, in contrast to subspecies *jamesii*, finely lobed ('crenulate'), but, as in subspecies *jamesii*, with an indistinct central pyrenoid. The two subspecies of *T. jamesii* also differ in their ITS rDNA sequences, which are available from Genbank (see Materials and Methods).

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