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# Photobiont diversity in lichens from metal-rich substrata based on ITS rDNA sequences

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ABSTRACT

The photobiont is considered as the more sensitive partner of lichen symbiosis in metal pollution. For this reason the presence of a metal tolerant photobiont in lichens may be a key factor of ecological success of lichens growing on metal polluted substrata. The photobiont inventory was examined for terricolous lichen community growing in Cu mine-spoil heaps derived by historical mining.

Sequences of internal transcribed spacer (ITS) were phylogenetically analyzed using maximum likelihood analyses. A total of 50 ITS algal sequences were obtained from 22 selected lichen taxa collected at three Cu mine-spoil heaps and two control localities. Algae associated with *Cladonia* and *Stereocaulon* were identified as members of several *Asterochloris* lineages, photobionts of cetrarioid lichens clustered with *Trebouxia hypogymniae* ined.

We did not find close relationship between heavy metal content (in localities as well as lichen thalli) and photobiont diversity. Presence of multiple algal genotypes in single lichen thallus has been confirmed.



## 1. Introduction

Through the world, there are many metal-polluted areas, including rocks and soils derived from metal mining. Specific lichen communities occurring on these substrata have been found (Nash, 1989; Purvis and Halls, 1996; Bačkor and Fahselt, 2004a; Banásová et al., 2006). Some lichens associated with heavy metal-rich substrata are common species able to tolerate metals. These lichens are frequent in both metal polluted as well as unpolluted areas. However, some lichen species are restricted to heavy-metal-rich substrata and their distribution reflects the presence of these substrata (Purvis and Halls, 1996).

Lichens growing in metal-rich environments are known to accumulate considerable amounts of heavy metals by their thalli. In the case of copper (Cu), lichen *Acarospora rugulosa* Körb. accumulates up to 16% on a dry weight basis (Chisholm et al., 1987). Lichens *Lecidea lactea* and *A. rugulosa* from cupriferous pyritic rocks in Central Scandinavia contained up to 5% of Cu on a dry weight basis (Purvis, 1984).

Although presence of Cu is essential for living organisms, like all other metals it is toxic at high concentrations. Lichen as a symbiotic unit possesses several mechanisms that detoxify harmful effects of metal excess in thalli. Exclusion of heavy metals is one of the most studied and effective processes related to heavy metal detoxification mechanisms in lichen thalli (Collins and Farrar, 1978). Cell walls of both bionts are included in metal exclusion; however, lichens, as a whole symbiotic unit, produce organic acids and lichen secondary metabolites, which can chelate metals. However, the extent of all these detoxification mechanisms is limited and excess metal can reach plasmalemma and enter the cells of lichen symbionts, where it is potentially toxic.

When grown aposymbiotically, axenic cultures of lichen algae (photobionts) seem to be more sensitive to excess heavy metals, e.g. Cu, than lichen fungi – mycobionts (Bačkor et al., 2006; 2007). For this reason, photobiont can be a likely key element of lichen sensitivity/tolerance as a symbiotic unit.

Physiological basis of metal detoxification in lichen is still poorly known; however photobiont cell walls, free amino acids, non-protein thiols and proteins (e.g. hsp70) have all been included in this process (see for review Bačkor and Fahselt, 2008). So far, many physiological and biochemical parameters were employed as markers for assessment of metal stress in lichen photobionts, including growth inhibition (Bačkor and

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Váczi, 2002), cytological effects (Tarhanen, 1998; Sanità di Toppi et al., 2005), enzymatic activities (Sanità di Toppi et al., 2004), assimilation pigment composition (Garty et al. 1992; Chettri et al., 1998), chlorophyll *a* fluorescence (Branquinho et al., 1997) and non-protein thiols (Pawlik-Skowrońska et al., 2002).

However, it has been found that even photobionts are differentially sensitive to presence of heavy metals in the environment. Photosynthesis of lichens containing cyanobacteria was more sensitive to the presence of Zn, Cd and Cu than that of lichens with eukaryotic photobionts (Brown and Beckett, 1983) and cyanobacterial *Nostoc* photobiont has been found to be more sensitive to Mn than eukaryotic *Dictyochloropsis* photobiont (Paul and Hauck, 2006). The sensitivity of eukaryotic photobionts also varies. For example, lipid metabolism has been found to be more affected by Cu and Pb in *Coccomyxa,* than in *Trebouxia* (Gushina and Harwood, 2006).

Photobiont involvement in lichen tolerance to heavy metals was suggested by Beck (1999), who found that all nine lichen species of the community Acarosporetum sinopicae on iron-rich rocks at "Schwarze Wand" (Austria) contained the same photobiont, *Trebouxia simplex* (reported as *Trebouxia jamesii* in Beck, 1999). However, further taxonomic study of metal tolerant photobionts is required, related to different chemical types of substrata, as well as their age and degree of ecological succession. Existence of metal-tolerant populations of lichen photobionts has been discovered within other taxa of *Trebouxia* photobionts. Evidence that this observed metal tolerance occurs in nature is supported by successful production of tolerance under laboratory conditions by gradually increasing, over a 3-year period, the Cu concentration of the medium. By this way a Cu-tolerant photobiont strain was obtained from wild-type *Trebouxia erici* (UTEX 911) (Bačkor and Váczi, 2002). When exposed to excess Cu the tolerant genotype exhibited uptake, growth rates, pigment content, membrane integrity, dehydrogenase activity, photosystem II activity, synthesis of free proline and non-protein thiols that were not significantly different from control photobionts growing on nutritional media (Bačkor and Fahselt, 2008).

In the present case, DNA sequence data provide us with the capability to evaluate photobionts in field-collected lichens. Diversity of lichen photobionts *in situ* has been studied using molecular markers, including internal transcribed spacer (ITS) region (e.g. Beck et al., 1998; 2002; Yahr et al., 2004; Hauck et al., 2007).

The main aim of this study was assessment of algal genotype preference by lichen fungi due to the presence of increased levels of heavy metals (mostly Cu) in specific, metal-rich copper mine-spoil heaps derived from the historical mining. Common lichen species, including members of genus *Cladonia*, *Cetraria* and *Stereocaulon*, were selected for this study as they grow in both heavy-metal-polluted as well as unpolluted habitats. In addition to Cu mine-spoil heaps, we chose for comparison two localities: first non-polluted by heavy metals and situated near the Cu-mining area in central Slovakia, and the second, rich in heavy metals (Cu content is however low here) but extrapolated from the Slovak localities and thus separated from the local pool of photobionts.

## 2. Material and methods

### 2.1. Collection of material

Specimens of lichens were collected during the years 2006 and 2007 at five different sites, three Cu mine-spoil heaps in central and eastern Slovakia, rocky slope in "Harmanec" in Slovakia (control) and former ore-sedimentation basin near "Chvaletice" in the Czech Republic (Table 1). All the lichen specimens are deposited in PL (Pilsen, Czech Republic). The present work did not involve humans or experimental animals.

### 2.2. Gelnica-Cechy, L'ubietová-Podlipa and Špania dolina

Three localities represent the Cu mine-spoil heaps derived from the historical mining activity situated in the mountain areas of central and eastern Slovakia: Volovské vrchy Mts. (Gelnica-Cechy, 500 m a.s.l, 48°50′N, 20°56′E), Slovenské Stredohorie Mts. (L'ubietová-Podlipa, 570–700 m a.s.l., 48°45′N, 19°22′E) and Low Tatra Mts. (Špania dolina, 780 m a.s.l., 48°49′N, 19°08′E). The area around L'ubietová and Špania dolina especially belonged to the most important mining centers of Slovakia and Europe. All the localities are strongly polluted by heavy metals, especially by Cu (concentrations of Cd, Co, Hg, Sb and other metals are also above the limit values). All the Cu mine-spoil heaps are more than 200 years old; recent human activity at this places is low; thus the substrate attributable to historical mining is the main source of metal pollution in the area. The mine heaps habitats are mostly colonized by a specific small group of vascular and non-vascular plants, including approximately 30 taxa of terricolous lichens, creating distinctive plant communities tolerant to heavy metals (Banásová et al., 2006).

### 2.3. Harmanec

Lichens were collected from soil and rocks at the rocky slope in Harmanec (48°49′N, 19°03′E), Vel'ká Fatra Mountains, central Slovakia, at approximately 500 m a.s.l.. This area belongs to Vel'ká Fatra National Park, and there is no known rock mineralization by Cu or other toxic metals.

### 2.4. Chvaletice

Lichens were collected in two parts of former industrial sedimentation basin near Chvaletice (250 m a.s.l.; 50°2′28.577″N, 15°26′39.361″E), East Bohemia: on clayey soil along the access road to the sedimentation basin and on dry soil directly in the sludge bed. Both of these sublocalities are strongly polluted by heavy metals; excess concentrations were measured especially for Fe, Mn, Zn, Al and Cd (Kovář, 2004). Except the heavy metal content, the soil in the sludge bed is characterized by very high salinity and extremely low pH (reaching as low as 3 in extreme cases). The ore-sedimentation basin was erected in 1952 for deposition of wastes from the factory producing sulfuric acid from pyrite ore. The basin was abandoned in 1979 and the ore deposit was colonized by pioneer, heavy metal resistant species of vascular plants, mosses and lichens (unsuccessful attempts to reforest the locality were conducted); 38 taxa of terricolous lichens were noted here by Palice and Soldán (2004) and Peksa (2009).

Lichens were identified using standard methods, including thin-layer chromatography (TLC) on Merck silica gel 60 F254 pre-coated glass plates in solvent systems A, B and C according to Orange et al. (2001).

### 2.5. Analysis of Cu content in soils and lichen thalli

Flame atomic absorption spectrometry (FAAS) was used to determine background Cu content in thalli of selected lichen species (*Cetraria islandica*, *Cladonia arbuscula*, *Cladonia mitis*, *Cladonia* cf. *novochlorophaea*, *Cladonia pyxidata* and *Cladonia rei*) growing on five selected localities.

Macroscopic foreign material adhering to lichen surfaces (e.g. soil particles) was removed with forceps and lichens were rinsed by deionized water. Lichens were dried at 80 °C for 24 h and 100 mg of dry material was digested for 48 h in 3 ml of concentrated $HNO_3$ (Suprapur, Merck, Darmstadt, Germany) and $H_2O_2$ (2:1, v/v) with the volume brought to 10 ml with deionized water, $n=3$ (Bačkor et al., 2007). Analysis of the trace elements was performed using a Perkin-Elmer 3030B spectrometer (Perkin-Elmer Corp., Norwalk, CT, USA). Each sample was analyzed at least three times and mean values were used as one observation.

Soil samples for determination of Cu content were collected from places where lichen thalli were collected. Three replicates were taken from each place. After removal of visible organic material and stones, soils were dried for 48 h at 80 °C and sieved through mesh with 0.8 mm pores. Total Cu was measured after digestion of 0.5 g DW in *aqua regia* (50 ml) for 24 h; solutions were then evaporated to dryness in a water bath and dissolved in 5% $HNO_3$ prior to measurement on FAAS. Detection was at Cu $\lambda_{max}$=324.8 nm.

### 2.6. DNA extraction, PCR amplification and DNA sequencing

Total genomic DNA was extracted from apical parts of lichen thalli following the standard CTAB protocol (Doyle and Doyle, 1987) with minor modifications, or with the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) with extraction buffers as recommended by the manufacturer. Algal DNA was resuspended in sterile $dH_2O$ and amplified by the polymerase chain reaction (PCR). The ITS1, ITS2 and 5.8S regions were amplified using the algal-specific primer nr-SSU-1780-5′ (5′-CTG CGG AAG GAT CAT TGA TTC-3′; Piercey-Normore and DePriest, 2001) and a universal primer ITS4-3′ (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al., 1990). All PCRs were performed in 20 μl reaction volumes (15.1 μl sterile Milli-Q Water,

**Table 1**
List of lichen taxa used in this study with collection informations and GenBank accession numbers of algal symbionts.

| Fungal taxa | Code of sample | Collection no. | Locality | GenBank |
|---|---|---|---|---|
| *Cetraria aculeata* | Backor 19 | Peksa 800 | Špania dolina | FM945343 |
| *Cetraria islandica* | Backor 07 | Peksa 799 | Špania dolina | FM945344 |
| *Cetraria islandica* | Backor 20 | Peksa 813 | Harmanec | FM945345 |
| *Cetraria islandica* | Backor 21 | Peksa 812 | L'ubietová | FM945346 |
| *Cladonia arbuscula* | Backor 13 | Peksa 789 | Gelnica | FM945347 |
| *Cladonia coccifera* | Clad 06 | Peksa 588 | Chvaletice | FM945351 |
| *Cladonia coccifera* | Clad 07 | Peksa 589 | Chvaletice | FM945352 |
| *Cladonia coccifera* | Backor 03 | Peksa 818 | Harmanec | FM945353 |
| *Cladonia coniocraea* | Clad 02 | Peksa 576 | Chvaletice | FM945354 |
| *Cladonia deformis* | Clad 08 | Peksa 918 | Chvaletice | FM945357 |
| *Cladonia fimbriata* | Backor 04 | Peksa 796 | Špania dolina | FM945358 |
| *Cladonia fimbriata* | Backor 27 | Peksa 815 | Harmanec | FM945359 |
| *Cladonia furcata* | Backor 08 | Peksa 797 | Špania dolina | FM945360 |
| *Cladonia furcata* | Backor 12 | Peksa 792 | Gelnica | FM945361 |
| *Cladonia furcata* | Backor 24 | Peksa 811 | L'ubietová | FM945362 |
| *Cladonia humilis* | Clad 11 A, B | Peksa 919 | Chvaletice | FM945348, FM945349 |
| *Cladonia humilis* | Clad 12 | Peksa 925 | Chvaletice | FM945350 |
| *Cladonia macilenta* | Clad 04 | Peksa 917 | Chvaletice | FM945363 |
| *Cladonia macilenta* | Clad 05 A, B, C | Peksa 922 | Chvaletice | FM945364, FM945365, FM945366 |
| *Cladonia mitis* | Backor 01 | Peksa 808 | L'ubietová | FM945367 |
| *Cladonia mitis* | Backor 05 | Peksa 807 | Špania dolina | FM945368 |
| *Cladonia* cf. *novochlorophaea* | Backor 06 | Peksa 798 | Špania dolina | FM945372 |
| *Cladonia ochrochlora* | Backor 02 | Peksa 816 | Harmanec | FM945369 |
| *Cladonia pleurota* | Backor 18 | Peksa 820 | Harmanec | FM945370 |
| *Cladonia pleurota* | Backor 28 | Peksa 810 | L'ubietová | FM945371 |
| *Cladonia pyxidata* | Backor 16 A, B | Peksa 791 | Gelnica | FM945373, FM945374 |
| *Cladonia pyxidata* | Backor 26 | Peksa 814 | Harmanec | FM945375 |
| *Cladonia rangiferina* | Backor 29 | Peksa 819 | Harmanec | FM945376 |
| *Cladonia rangiformis* | Backor 15 | Peksa 790 | Gelnica | FM945377 |
| *Cladonia rei* | Clad 16 A, B | Peksa 927 | Chvaletice | FM945355, FM945356 |
| *Cladonia rei* | Clad 09 | Peksa 921 | Chvaletice | FM945378 |
| *Cladonia rei* | Backor 14 | Peksa 787 | Gelnica | FM945380 |
| *Cladonia rei* | Backor 23 A, B | Peksa 794 | Špania dolina | FM945381, FM945382 |
| *Cladonia rei* | Backor 22 A, B | Peksa 809 | L'ubietová | FM945386, FM945387 |
| *Cladonia subulata* | Clad 10 | Peksa 926 | Chvaletice | FM945379 |
| *Cladonia subulata* | Clad 13 A, B | Peksa 916 | Chvaletice | FM945383, FM945384 |
| *Cladonia subulata* | Clad 14 | Peksa 924 | Chvaletice | FM945385 |
| *Cladonia* sp. | Clad 15 | Peksa 920 | Chvaletice | FM945388 |
| *Diploschistes muscorum* | Dip 08 | Peksa 923 | Chvaletice | FM945389 |
| *Diploschistes muscorum* | Dip 09 | Peksa 928 | Chvaletice | FM945390 |
| *Stereocaulon* sp. | Backor 09 | Peksa 801 | Špania dolina | FM945392 |
| *Stereocaulon tomentosum* | Backor 10 | Peksa 786 | Gelnica | FM945391 |

2 μl 10′ PCR buffer (Sigma), 0.4 μl dNTP (10 μM), 0.25 μl of primers (25 pmol/ml), 0.5 μl Red Taq DNA Polymerase (Sigma) (1U/ml), 0.5 μl of $MgCl_2$ and 1 μl of DNA (not quantified)).

PCR and cycle-sequencing reactions were performed in either an XP thermal cycler (Bioer) or a Touchgene gradient cycler (Techne). PCR amplification of the algal ITS began with an initial denaturation of 95 °C for 5 min, and was followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Identical conditions were used for the amplification of the actin I locus, except that an annealing temperature of 60–62 °C was used. The PCR products were quantified on 1% agarose gel stained with ethidium bromide and cleaned either with the JetQuick PCR Purification Kit (Genomed) or with QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's protocols. The purified amplification products were sequenced from both directions with the PCR primers at Macrogen, Inc. (Seoul, Korea, http://dna.macrogen.com) and submitted to GenBank (accession numbers in Table 1). Each polymorphic position was checked manually in all electropherograms. Sequences of ITS variants were reconstructed from the sequences containing ambiguities following Clark (1990) and Beszteri et al. (2005). The method of manual checking of polymorphic sites (Clark, 1990) has been recently utilized by Beszteri et al. (2005), who used the polymerase in PCR reactions. Contrary to Clark (1990) we do not identify haplotypes in diploid population, but distinguish particular species co-occurring in the lichen thallus. First, sequences without any ambiguities were selected, as these undoubtedly represent sequence variants occurring in the sample. Then those containing a single ambiguous position were resolved as two variants differing at the single position concerned and the resulting variants were added to the list of resolved variants. For each variant thus identified, the remaining sequences containing more than one ambiguities were screened. If the known variant could be made from some combination of the ambiguous sites, the complement of the variant was recovered as another potential variant. If only a single variant or a single variant and its complement were found for a sequence containing multiple ambiguities in this way, the variants were resolved unambiguously, and the complementary variant was added to the list of resolved variants. This was repeated as long as all ambiguities were resolved.

### 2.7. Sequence alignment and phylogenetic analyses

*Asterochloris* and *Trebouxia* ITS sequences (comprising ITS1, 5.8S and ITS2 regions) were aligned on the basis of their rRNA secondary structure information (Beiggi and Piercey-Normore, 2007) with MEGA 3 (Kumar et al., 2004). Using RNA secondary structure as a guide in aligning rRNA sequences is widely used. The advantage of this approach is in apparent improvement of hardly alignable regions. There are no conflicts between the primary and secondary structure alignment. With the aid of secondary structure information, we are able to align undoubtedly even highly variable parts of the alignment. Positions with deletions in a majority of sequences were removed from the alignment, resulting in an alignment comprising 523 (*Asterochloris*) and 607 (*Trebouxia*) base positions, respectively. The phylogenetic trees were inferred by maximum likelihood (ML) and weighted parsimony (wMP) criteria using PAUP*, version 4.0b10 (Swofford, 2003), and by Bayesian inference (BI) using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). A substitution model was estimated using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander, 2004). Accordingly, the HKY+I+Γ model was chosen for *Asterochloris* alignment, whereas

in *Trebouxia*, GTR+I+Γ model was deemed the best. Maximum likelihood analyses consisted of heuristic searches with 1000 random sequence addition replicates and tree bisection reconnection swapping. Reliability of the resulting topology was tested using bootstrap analysis (100 replications) consisting of heuristic searches with 10 random sequence addition replicates, tree bisection reconnection swapping and a rearrangement limit of 5000 for each replicate. The wMP bootstrapping was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10,000 for each replicate) and gap characters treated as missing data. In BI analysis, the datasets were partitioned into stem and loop regions, and into ITS1, ITS2 and 5.8 rRNA partitions. Different substitution models were then selected for the six partitions. For the loop regions a 4-state, single-nucleotide substitution model was selected, while for the paired stem regions, the doublet model (a 16-state RNA stem substitution model) was chosen (Verbruggen and Theriot, 2008). According to our results, the Bayesian analyses without using the doublet model have considerable lower posterior probabilities of internal branches. In the analysis of ITS rDNA sequences acquired from 60 *Asterochloris* sequences, the number of nodes receiving high/moderate PP support decreased from 11/7 to 10/4, without using the partitioned dataset with doublet model.

Substitutions models for rRNA partitions were estimated using the Akaike Information Criterion (AIC) as follows: GTR+Γ for ITS1, K80+I+Γ for ITS2 and JC for 5.8 rRNA. Two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains employing the above-stated evolutionary model. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and burn-in was determined using the "sump" command.

### 2.8. Statistical analysis

One-way analysis of variance and Tukey's pairwise comparisons (MINITAB Release 11, 1996) were applied to determine the significance ($P<0.05$) of Cu content in lichens and soils.

## 3. Results

Cu content in lichens was the highest in samples collected from Cu mine-spoil heaps in Špania dolina (Table 2). Lichens collected in Gelnica and L'ubietová-Podlipa also contained significantly higher content of Cu when compared to those collected in Harmanec and Chvaletice. Lichens *C.* cf. *novochlorophaea* and *C. pyxidata* collected from Cu mine-spoil heaps in Špania dolina and Gelnica were the most effective in entrapment of soil particulates from substrate and contained the highest content of Cu when compared with the rest of lichens analyzed for Cu content. Cu accumulation in lichens reflected Cu availability in substrata (Table 2).

We examined the samples of 23 terricolous lichen taxa predominantly with fruticose thalli collected at five investigated localities. A total of 50 ITS algal sequences were obtained (Table 1).

The photobionts obtained from specimens of *Cladonia*, *Diploschistes* and *Stereocaulon* (46 samples) were established to be a member of the genus *Asterochloris* (Fig. 1). The phylogram inferred from *Asterochloris* sequences contains four well supported lineages (A–D) and some additional strains with unsupported phylogenetic position (containing samples Backor 10, 23B and further related sequences). The clades A–D include 34 from a total of 46 *Asterochloris* sequences of photobionts associated altogether with 16 fungal species. We found out rather low degree of algal specificity. No clade was identified as specific to particular fungal species; indeed, all lineages were associated with two or more mycobionts.

In the remaining four samples representing cetrarioid lichens (*Cetraria aculeata*, *C. islandica*), photobiont belonging to the genus *Trebouxia* (s. str.) was detected. Sequences were genetically closely related, and clustered with *Trebouxia hypogymniae* Hauck and Friedl ined. (Hauck et al., 2007). The ML phylogram revealed two well-resolved lineages within *T. "hypogymniae"*, both containing photobionts of lichens growing in Cu mine-spoil heaps (Fig. 2).

Analyzing photobiont diversity in various mycobiont species (Table 3), different degrees of selectivity toward photobiont lineages were detected. In some fungal species, only single photobiont clade was found, even though lichens from more than one locality were investigated: *T. hypogymniae* ined. in *C. islandica*; *Asterochloris* clade A in *C. mitis* and *Cladonia pleurota*, clade D in *Cladonia furcata*. On the other hand, in the fungal species *Cladonia humilis*, *Cladonia macilenta*, *C. pyxidata*, *C. rei* and *Cladonia subulata*, two, three or four associated photobiont lineages were recorded. Moreover, in some specimens of former five lichen taxa, more than one photobiont was identified in a single thallus (podetium). Usually, these photobionts were established as members of the distantly related *Asterochloris* lineages (e.g. photobionts of *C. macilenta* Clad 05 belong to clades A and C; Fig. 1). To confirm the primary findings of multiple algal genotypes, we analyzed photobiont diversity in different parts of *C. macilenta* and *C. subulata* thalli (clump): small pieces of thalli from the tips of one younger and one older podetia growing side by side and from basal squamules on the base of these podetia were selected (in previous analysis, DNA was extracted from the whole podetium). In both fungal species, only a single photobiont ITS variant was detected in each sample. Different photobionts were recorded for the tips of neighboring podetia, the basal squamules contained the same photobiont found in younger podetium.

We did not find distinct differences in diversity of photobionts among the localities. The number of photobiont lineages was similar in areas with different heavy metal contents. Lineages of *Asterochloris* occurring in three Cu polluted habitats (clades A–C) were found also in Chvaletice, where the Cu content is very low (but the amount of other metals is high). Moreover, the majority of photobionts occurring in metal polluted localities were detected also in natural habitat without distinct heavy metal pollution in Harmanec (clades A–C and *T. hypogymniae* ined.). The lack of particular clades in certain localities may be caused only by incomplete sampling (we chose only part of lichen taxa growing in studied sites for our approach). For example, *Asterochloris* clade D was found only in sedimentation basin in Chvaletice and it is missing in all four Slovak localities; however, we found it in the thalli of *Lepraria borealis* collected in northeast

**Table 2**
Cu content (μg/g) in selected lichen species and soils collected at five localities; H=Harmanec, SD=Špania dolina, L=L'ubietová-Podlipa, G=Gelnica, CH=Chvaletice.

| | H | SD | L | G | CH |
|---|---|---|---|---|---|
| *Cu content – lichens* | | | | | |
| *Cetraria islandica* | 2.98 ± 1.67 | 64.4 ± 18.7 | 24.3 ± 8.25 | – | – |
| *Cladonia arbuscula* | – | – | – | 28.5 ± 11.4 | – |
| *C. mitis* | – | 66.5 ± 20.6 | 27.6 ± 11.2 | – | – |
| *C.* cf. *novochlorophaea* | – | 242 ± 46.3 | – | – | – |
| *C. pyxidata* | 3.94 ± 0.48 | – | – | 163 ± 72.8 | – |
| *C. rei* | – | – | – | – | 3.28 ± 0.25 |
| Cu content – soils | 45.2 ± 9.36 | 1368 ± 161 | 924 ± 242 | 1486 ± 457 | 18 ± 6.2 |

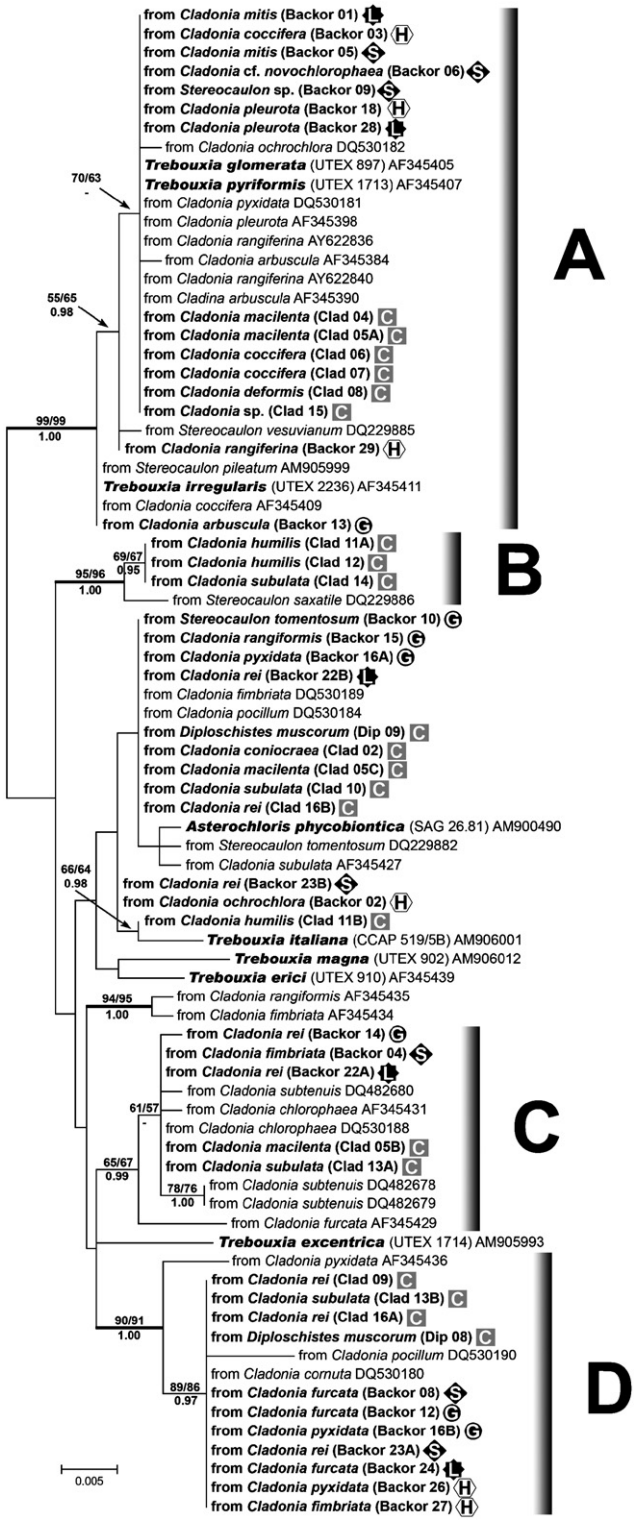


**Fig. 1.** ML phylogram of *Asterochloris* algae based on ITS rDNA sequences using a HKY+I+Γ model. Values at the nodes indicate statistical support estimated by three methods – maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick branches represent nodes receiving high statistical support in at least two bootstrap/posterior probability analyses. ITS sequences determined in this study are given in bold face. Strain affiliation to four lineages (A–D) is indicated. Localities in which algal strains were found are illustrated by the symbols following the strain name (C – Chvaletice, G – Gelnica-Cechy, H – Harmanec, L – L'ubietová-Podlipa, S – Špania dolina). Different colors of symbols indicate degree of polution (locality unpolluted by heavy metals are given in white, Cu mine-spoil heaps in black and polluted locality with low Cu content in gray). Scale bar – substitutions per site.

Low Tatra Mts. (Slovakia), not so far from the investigated sites (unpublished results).

## 4. Discussion

### 4.1. Photobiont identity

Phylogram inferred from *Asterochloris* sequences (Fig. 1) contains only one lineage including our samples (clade A), which corresponds to a formally described morphospecies (*Trebouxia glomerata*, *Trebouxia irregularis* and *Trebouxia pyriformis* – affiliation of these species to the genus *Asterochloris* was confirmed by several studies, e.g. Piercey-Normore and DePriest, 2001 – our clade A corresponds well to clade I in their paper). Additional lineages do not have any affiliation to yet described species; however, all of them were previously reported by other authors within phylogenetic analysis of various lichen photobionts (e.g. Yahr et al., 2004; Beiggi and Piercey-Normore, 2007). Based on neighbor-joining analysis (unpublished results) performed using all known "*Asterochloris*" sequences (219 sequences obtained from GenBank) and our 46 sequences, we tried to find out the size (number of including sequences) and characters of particular clades. Clade A was the most frequently occurring clade (91 algal sequences from almost 50 lichen taxa); other well supported clades contain fewer sequences (B – 11 sequences/10 lichen taxa, C – 12/8 and D – 14/10).

Similar to *Asterochloris* clades, *T. hypogymniae* Hauck and Friedl ined. is known from various lichen taxa, especially from families Parmeliaceae and Umbiliariaceae. It is closely related to the phenotypic species *Trebouxia angustilobata* (A. Beck) A. Beck ined. (syn. *T. jamesii* (Hildreth and Ahmadjian) Gärtner subsp. *angustilobata* A. Beck; Beck, 1999; Beck et al., 2002) and an undescribed species *Trebouxia* "*vulpinae*" (Kroken and Taylor, 2000). Moreover, these sequences are related to *T. simplex* Tschermak–Woess (photobiont sequence AJ51135, AJ511354 from *Lecanora conizaeoides* clustered in analysis of Hauck et al. (2007) with sequence of the type strain of *T. simplex*) and another undescribed photobiont of *Letharia* species *T.* "*letharii*" (Kroken and Taylor, 2000). All these taxa form a big clade that seems to be similar to the variously marked clades in several published works: "*T. jamesii* complex" of Kroken and Taylor, 2000, clade A of Opanowicz and Grube (2004), clade S3 of Blaha et al. (2006), clade "*T. jamesii*" of Piercey-Normore (2006) and clade 1 in phylogenetic analysis of Hauck et al. (2007).

### 4.2. Photobionts and heavy metal pollution

Due to the low technology of mining operations in medieval times, the Cu content of the mine-spoil heaps is still very high (Bačkor and Fahselt, 2004a; Banásová et al., 2006) – at the localities in central Slovakia it may reach more than 3600 mg/kg, the limit value for non-contaminated soils established by the Slovakian Ministry of Environment is 36 mg/kg (Banásová et al., 2006). Results of soil analyses in the present study (Table 2) revealed that Cu soil content is significantly higher in samples from Cu mine-spoil heaps in Špania dolina, L'ubietová-Podlipa and Gelnica when compared with soil samples collected in Harmanec and Chvaletice.

The effectiveness of lichens in intercepting atmospheric particulates (usually up to 100 μm), as well as soil particles from their substrate, has been shown in many studies (Loppi et al., 1999 and references therein). These particles may be simply deposited onto the lichen surface or trapped in the intercellular spaces of the medulla (Garty, 2001) and can remain unaltered for

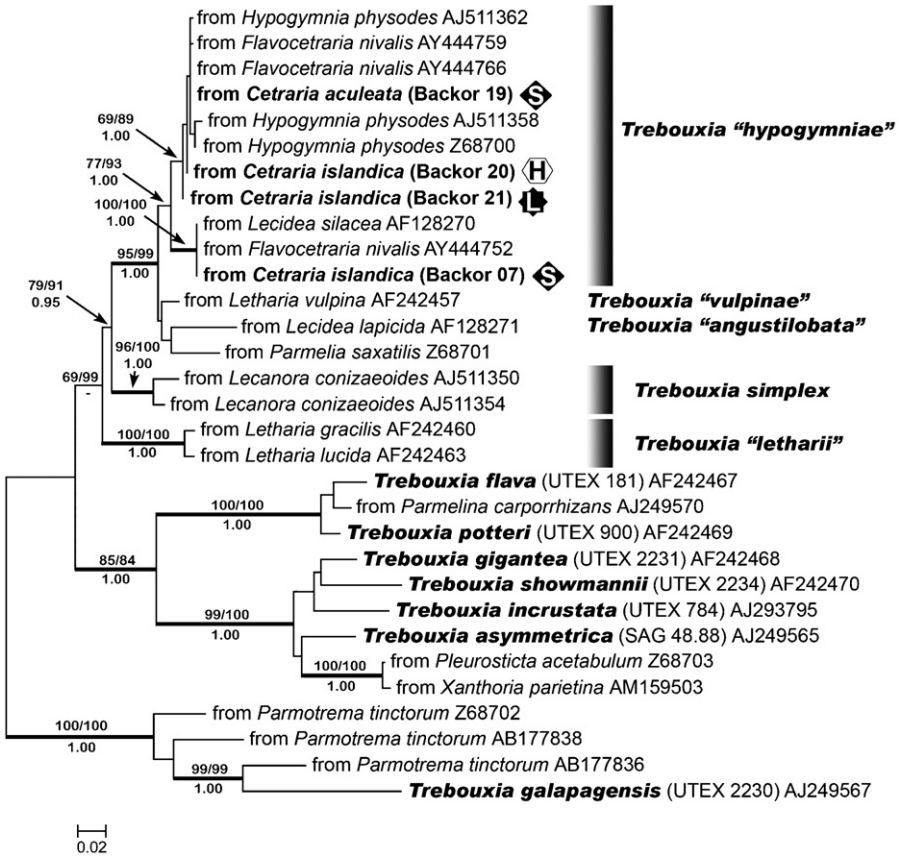


**Fig. 2.** ML phylogram of *Trebouxia* algae based on ITS rDNA sequences using a GTR+I+Γ model. Values at the nodes indicate statistical support estimated by three methods – maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick branches represent nodes receiving high statistical support in at least two bootstrap/posterior probability analyses. ITS sequences determined in this study are given in bold face. Localities in which algal strains were found are illustrated by the symbols following the strain name (see Fig. 1). Scale bar – substitutions per site.

a long time. It has been demonstrated previously that some lichens can accumulate metals in considerable amounts, reaching more than 5% dry weight (Seaward, 1973; Purvis, 1984; Bačkor and Fahselt, 2004a). Although in the present study we found that lichens growing on Cu mine-spoil heaps accumulated significantly higher amount of Cu when compared with control localities, Cu concentrations determined in lichens were much lower when compared with extreme amounts of some metals previously found in lichens with crustose morphology and lichens from heavily polluted areas, where atmosphere was the principal source of metal pollution. This is probably due to very low atmospheric deposit of heavy metals and the morphology of lichen thalli of investigated taxa. Although substrate (Cu mine-spoil heaps) is still rich in Cu, lichens from genera *Cetraria* and *Cladonia* are attached to the substrate by only a limited surface area. Significantly higher Cu content has been determined in lichens *C.* cf. *novochlorophaea* and *C. pyxidata*. Due to morphological properties these taxa are much stronger associated with substrate, which leads to higher entrapment of soil particulates. Cu content in this lichen was comparable with morphologically similar lichen *C. pleurota* growing on metal rich soils near Sudbury (Ontario, Canada), as has been previously demonstrated by Bačkor and Fahselt (2004b).

The relationship between the occurrence of particular photobiont species and the amount of Cu in lichen thallus was not confirmed. We found the same photobionts in thalli with high and low amount of Cu. In the analyzed samples *C.* cf. *novochlorophaea* and *C. mitis* collected in Špania dolina, the same photobiont (*Asterochloris* clade A) was found, though thallus of *C.* cf. *novochlorophaea* contained four times higher Cu content than that of *C. mitis* (see Table 2); *C. pyxidata* from Gelnica (Backor 16A) with Cu content of about 163 μg/g has the same algae as *C. rei* (Clad 16B) from Chvaletice with 3.28 μg/g of Cu. We did not detect differences between lichens with shrubby thalli poorly contacted to substrate and more closely attached lichens like *C. pyxidata*. The same *Asterochloris* clades contained for example *C. mitis* together with *C. coccifera*, *C. pleurota*, etc. (clade A) and *C. furcata* with *C. rei*, *C. fimbriata*, etc. (clade D).

Similarly, there were no distinct differences in diversity of photobionts from localities with high and low amounts of heavy metals. From the 5 well resolved algal lineages occurring in heavy-metal rich sites, 3 lineages were detected in unpolluted locality Harmanec (the same photobiont was also detected in *C. rei* from Špania dolina (Backor 23B) and *Cladonia ochrochlora* from Harmanec (Backor 02)). Moreover, we can reject the specific influence of Cu on photobiont community composition. In Chvaletice, where the Cu content is not considerable, we found all lineages known from Cu mine-spoil heaps.

Based on our results, we can say that the photobiont inventory of heavy metal polluted habitats is rather rich and without species specific only to those habitats. There are two possible explanations for these results: (1) the highest concentrations of heavy metals measured in thalli of investigated lichens are not high

**Table 3**
Selectivity of fungal taxa – number of photobiont lineages associated with particular fungal taxa for five investigated localities (weakly supported *Asterochloris* lineages were counted as one lineage).

| | No. of photobiont lineages | No. of localities | No. of samples analyzed |
|---|---|---|---|
| *Cladonia subulata* | 4 | 1 | 4 |
| *Cladonia rei* | 3 | 4 | 8 |
| *Cladonia macilenta* | 3 | 1 | 5 |
| *Cladonia fimbriata* | 2 | 2 | 2 |
| *Cladonia humilis* | 2 | 1 | 2 |
| *Cladonia pyxidata* | 2 | 2 | 3 |
| *Diploschistes muscorum* | 2 | 1 | 2 |
| *Cetraria aculeata* | 1 | 1 | 1 |
| *Cetraria islandica* | 1 | 3 | 3 |
| *Cladonia arbuscula* | 1 | 1 | 1 |
| *Cladonia* cf. *novochlorophaea* | 1 | 1 | 1 |
| *Cladonia coccifera* | 1 | 1 | 3 |
| *Cladonia coniocraea* | 1 | 1 | 1 |
| *Cladonia deformis* | 1 | 1 | 1 |
| *Cladonia furcata* | 1 | 3 | 3 |
| *Cladonia mitis* | 1 | 2 | 2 |
| *Cladonia ochrochlora* | 1 | 1 | 1 |
| *Cladonia pleurota* | 1 | 2 | 2 |
| *Cladonia rangiferina* | 1 | 1 | 1 |
| *Cladonia rangiformis* | 1 | 1 | 1 |
| *Cladonia* sp. | 1 | 1 | 1 |
| *Stereocaulon* sp. | 1 | 1 | 1 |
| *Stereocaulon tomentosum* | 1 | 1 | 1 |

enough to considerably affect photobionts or (2) all detected photobiont lineages are tolerant to high heavy metal content (as well as associated mycobionts). The second explanation seems to be more probable.

The major part of investigated lichens (lichen-forming fungi) represents the common inhabitants of open sunny sites. In comparison with the results of Banásová et al. (2006), all lichens collected on Cu mine-spoil heaps seem to be common in these biotopes. However, some of these lichen taxa belong to very common pioneer lichens, growing in a wide spectra of habitats, including anthropogenic sites (e.g. *C. pyxidata*, *C. rei*), others (e.g. *C. islandica*, *C. aculeata*, *C. pleurota*, *C. furcata*) grow commonly also in natural or seminatural habitats not contaminated by heavy metals, especially on sandy soils or rocky/scree slopes (e.g. Harmanec).

Photobiont lineages occurring in natural habitat (Harmanec) were found also in heavy-metal-polluted localities; thus we can suggest that they are tolerant to heavy metals. However, using additional photobiont sequences obtained from GenBank we revealed further data on the ecology of studied photobiont lineages. We found that these photobionts are also tolerant to other extremes of environmental factors. For example, *Asterochloris* clade D contains algae very tolerant to different pH as well as climatic conditions. There are samples obtained from completely dissimilar conditions: samples from sedimentation basin in Chvaletice with extremely low pH, situated in warm lowland in European temperate belt, together with two samples (DQ530180, DQ530190) collected in highly calcareous area with arctic climate in Manitoba, North America (Michelle Piercey-Normore, pers. comm.). Algae from *A.* clade C are probably common on varying toxic substrates, because besides our samples from heavy metal rich localities there are also two samples from *C. subtenuis* collected in serpentine area (DQ482678, DQ482680; Rebecca Yahr, pers. comm.). In addition to these examples, there are many another published sequences within our clades not shown in the phylogenetic tree in Fig. 1 (especially in very large *A.* clade A), which originate from different continents, habitats and lichens. Thus, we found *Asterochloris* clades that represent very successful lineages with broad ecological amplitude (including tolerance to heavy metals) and worldwide distribution. *T. hypogymniae* ined. also fulfils such a definition. It was identified in lichens from very varying conditions. Besides our samples from heavy metal contaminated areas, it is known also from European specimens of *Lecidea lapicida* and *Lecidea silacea* (Beck, 1999) and antarctic *Umbilicaria* samples (Romeike et al., 2002), all growing on iron rich rocks (laboratory test confirmed high tolerance of this species to iron; Beck, 1999). Additional photobiont sequences within *T. hypogymniae* originate from various taxa of epiphytic lichens growing in diverse conditions (see references in capture Photobiont identity) and also from *Flavocetraria nivalis* collected from lowland to arctic/alpine habitats (Opanowicz and Grube, 2004).

In addition to the mentioned ecological factors (climate, pH, toxicity of substrate), the success of a photobiont species is dependent also on the level of its specificity to potential symbiotic partners. We detected very low specificity of *Asterochloris* lineages as well as of *T. hypogymniae*, each clade was associated with at least eight fungal species. The broad ecological amplitude, including the low specificity to fungal partners, provides flexibility of the photobiont to environmental changes and the ability to colonize various types of habitats.

### 4.3. Fungal selectivity and multiple algal genotypes

The degree of selectivity to photobionts is different by various species of lichenized fungi (e.g. Piercey-Normore, 2004; Yahr et al., 2006). Simultaneously, several studies demonstrated some ecological preferences of particular photobiont species (Beck, 1999; Beck et al., 2002; Helms, 2003; Blaha et al., 2006, Guzow-Krzemińska, 2006; Hauck et al., 2007). Therefore, similar to the photobionts, mycobionts with low selectivity should be able to colonize more wide spectra of substrates and habitats, because they can associate with different photobionts occurring in various conditions.

Most of the lichens growing on man-made substrates like Cu mine-spoil heaps belong to common species and the lichen communities in these habitats use to be more or less similar. We investigated (if it was possible) the same lichen taxa from other localities to find out the degree of selectivity in particular fungal species. Moreover, we added some accessible photobiont sequences of investigated lichen taxa from GenBank (Figs. 1 and 2). Although the results are influenced by low number of investigated samples, it is possible to observe distinct differences in mycobiont selectivity in various lichen taxa (Table 3). We found high degree of selectivity in cup lichens from the *Cladonia coccifera* group (*C. coccifera*, *C. pleurota*), which associated only with *Asterochloris* clade A similar to reindeer lichens *C. arbuscula*, *C. mitis* and *Cladonia rangiferina*. *C. furcata* from Slovak samples is highly selective to *Asterochloris* clade D; however, there is also one sample from Karelia (AF 345429) in clade C. On the other hand, several fungal species exhibited rather low selectivity (3 or 4 possible photobionts). All these taxa belong to pioneer lichens, growing in various conditions, including urban areas (*C. humilis*, *C. pyxidata*, *C. rei*, *C. subulata*, etc.). The ability of fungi to switch photobionts is definitely one of the reasons why these lichens are very successful in colonizing different types of substrates and habitats. We can find similar examples also among crustose lichens associated with *Trebouxia* photobionts, e.g. euryecious taxa *Protoparmeliopsis muralis* (Guzow-Krzemińska, 2006) and *Lecanora rupicola* (Blaha et al., 2006).

One of the expressions of low mycobiont selectivity is evidently also the occurrence of multiple algal genotypes in a single lichen thallus. We found two photobiont lineages in one thalli (podetium) of four *Cladonia* species; in *C. macilenta* we detected even three different photobiont lineages in the same lichen tissue. All these lichens are characterised by low selectivity level (Table 3).

The occurrence of more than one algal genotypes in a single thallus was previously reported by several authors (see the discussion in Nelsen and Gargas, 2009). Unfortunately, the development of the thalli with multiple photobionts is not quite elucidated. Particular podetia may arise from a large number of soredia originating from various thalli (Schuster et al., 1985; Jahns, 1988; Honegger, 1992). Each soredium can contain different photobionts as well as mycobiont genotypes and therefore the resulting thallus can be a mix of multiple genotypes of both symbionts. In another case, the additional photobiont (as well as mycobiont) can be incorporated to the original thallus during its growth, when the foreign propagule lands on the thallus surface (Piercey-Normore, 2006). Similar possibility was suggested by Ohmura et al. (2006) as a fusion of two different individual thalli. Both explanations may be possible in cases of *Cladonia* samples. However, the analysis of small pieces of thalli revealed only a single photobiont in each sample (similar phenomenon was observed by Helms et al. (2001) in *Rinodina* and *Rinodinella* species). The finding of only a single photobiont in basal part of thalli (squamules) supports the theory of the additional adoption of another alga. The soredia of particular podetia containing different photobionts (samples of *C. macilenta* Clad 05A and C, *C. subulata* Clad 13A and B) may attach to neighboring podetia within a clump and may then be incorporated to the thallus (however, imported soredia from another lichen species might be in some cases attached only onto the surface of lichens without incorporation). If the incoming soredia contain more suitable and adapted photobiont, the mycobiont can theoretically completely switch original photobiont for the new compatible algae. The algal switching within the mature thallus was described by Friedl (1987) in partially parasitic lichen *Diploschistes muscorum*. However, we did not find multiple algal genotypes in our investigated samples of this lichen and thus we cannot confirm his results.

### 4.4. Photobionts in lichen communities

Cu mine-spoil heaps investigated in the present work are between 100 and 300 years old, and mainly at the high end of this range (Bačkor and Fahselt, 2004a). During this time, stable and species rich lichen communities have been developing there (about 25 terricolous fruticose lichens including 19 *Cladonia* species – Banásová et al., 2006; our data). By contrast, ore-sedimentation basin in Chvaletice was abandoned about 30 years ago and only 10 pioneer *Cladonia* species colonized this area during the spontaneous succession. However, the number of algal species and composition of photobiont community are almost identical in both types of habitats. Almost all *Asterochloris* lineages are common to Cu heaps as well as Chvaletice sedimentation basin. Based on these results, we can assume that the colonization of completely new habitats by photobionts and establishment of local photobiont pool proceeds relatively very fast and afterwards it is more or less stable in time. This later stability is probably caused by specific (extreme) conditions in the habitat, which preclude the growth of another (less tolerant) photobiont species.

Although *Trebouxia* (including *Asterochloris*) photobionts are rare outside thalli (Ahmadjian, 2004), these algae may belong to first settlers of newly developed habitats, for example areas previously completely sterilized by a forest fire (Mukhtar et al., 1994) or Cu heaps derived by Cu mining. However, as *Trebouxia* cells outside lichen thalli may be only short-living, we assume that mostly vegetatively reproducing lichens are able to bring new photobiont species into the newly developed habitats. The photobiont transfer to the new biotope of the abandoned sedimentation basin in Chvaletice was probably provided by vegetative propagules of pioneer lichens growing here recently. Due to their low selectivity and a broad spectrum of compatible photobionts, a few fungal species could bring the considerable number of algal species. For example, only three lichens *C. macilenta*, *C. rei* and *C. subulata* are theoretically able to transfer five *Asterochloris* lineages. Moreover, these lichens represent the most common *Cladonia* species occurring in the wide range of habitats and substrates. Thus, together with other similar species like *C. humilis*, *C. fimbriata*, *C. pyxidata*, etc., they probably represent very important photobiont transferring system. Their numerous asexual propagules may consequently serve as the photobiont source for other fungal species incoming to the habitat via sexual as well as asexual diaspores, which results in the sharing of photobionts by several lichens at one locality. Photobiont sharing was previously observed several times (Beck et al., 1998; Romeike et al., 2002; Doering and Piercey-Normore, 2009). Although we investigated photobionts from a low number of fungal species in particular localities, we observed the sharing of photobionts in several cases, for example *C. mitis*, *C.* cf. *novochlorophaea* and *Stereocaulon* sp. share *Asterochloris* clade A in Špania dolina; *C. rei*, *C. subulata* and *D. muscorum* share *A.* clade D in Chvaletice.

## 5. Conclusions

Many studies confirmed the harmful effect of high concentration of some heavy metals (Cd, Cu, Ni, etc.) to lichen photobionts (Bačkor and Fahselt, 2008). Therefore, mainly the most tolerant of them could be frequent in habitats with high heavy metal content. As lichen photobionts are mentioned as sensitive to heavy metals (Ahmadjian, 1993), we therefore expected to find low species diversity in such habitats (occurrence of a low number of specific species). However, we found here rather high number of photobionts – using ITS rDNA sequences we detected 5 well resolved and other weakly supported clades within only 50 lichen samples from 5 localities. We found out that these clades represent photobiont lineages with broad ecological amplitude and worldwide distribution. There is a discrepancy with the results of Beck (1999) who found only two photobiont taxa in the community of crustose lichens on iron-rich rock. However, crustose lichens are more tightly attached to substrate and therefore probably much more influenced by its character. In our case, the harmful effect (concentration) of heavy metals can be low to considerably affect photobionts, especially due to the fruticose thalli, which is only poorly associated with toxic substrate. Therefore, further study will include investigation of photobiont diversity in crustose lichens growing at Cu mine spoil heaps.

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## References

Ahmadjian, V., 1993. In: The Lichen Symbiosis. John Wiley, New York.
Ahmadjian, V., 2004. *Trebouxia*: reflections on a perplexing and controversial lichen photobiont. In: Seckbach, J. (Ed.), Symbiosis, Mechanisms and Model Systems. Springer, Amsterdam, pp. 373–383.
Bačkor, M., Fahselt, D., 2004a. Using EDX-micronalysis and X-ray mapping to demonstrate metal uptake by lichens. Biologia 59, 39–45.
Bačkor, M., Fahselt, D., 2004b. Physiological attributes of the lichen *Cladonia pleurota* in metal-rich and control sites near Sudbury (Ontario, Canada). Environ. Exp. Bot. 52, 149–159.
Bačkor, M., Fahselt, D., 2008. Lichen photobionts and metal toxicity (review article). Symbiosis 46, 1–10.
Bačkor, M., Váczi, P., 2002. Copper tolerance in the lichen photobiont *Trebouxia erici* (Chlorophyta). Environ. Exp. Bot. 47, 11–20.
Bačkor, M., Pawlik-Skowrońska, B., Tomko, J., Buďová, J., Sanità di Toppi, L., 2006. Response to copper stress in aposymbiotically grown lichen mycobiont *Cladonia cristatella*: uptake, viability, ergosterol and production of non-protein thiols. Mycol. Res. 110, 994–999.
Bačkor, M., Pawlik-Skowrońska, B., Buďová, J., Skowroński, T., 2007. Response to copper and cadmium stress in wild-type and copper tolerant strains of the lichen alga *Trebouxia erici*: metal accumulation, toxicity and non-protein thiols. Plant Growth Regul. 52, 17–27.
Banásová, V., Horák, O., Čiamporová, M., Nadubinská, M., Lichtscheidl, I., 2006. The vegetation of metalliferous and non-metalliferous grasslands in two former mine regions in Central Slovakia. Biologia 61, 433–439.
Beck, A., 1999. Photobiont inventory of a lichen community growing on heavy-metal-rich rock. Lichenologist 31, 501–510.
Beck, A., Friedl, T., Rambold, G., 1998. Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. New Phytol. 139, 709–720.
Beck, A., Kasalicky, T., Rambold, G., 2002. Myco-photobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. New Phytol. 153, 317–326.
Beiggi, S., Piercey-Normore, M.D., 2007. Evolution of ITS ribosomal RNA secondary structures in fungal and algal symbionts of selected species of *Cladonia* sect. *Cladonia* (Cladoniaceae, Ascomycotina). J. Mol. Evol. 64, 528–542.
Beszteri, B., Ács, É., Medlin, L.K., 2005. Ribosomal DNA sequence variation among sympatric strains of the *Cyclotella meneghiniana* complex (Bacillariophyceae) reveals cryptic diversity. Protist 156, 317–333.
Blaha, J., Baloch, E., Grube, M., 2006. High photobiont diversity in symbioses of the euryoecious lichen *Lecanora rupicola* (Lecanoraceae, Ascomycota). Biol. J. Linn. Soc. 88, 283–293.
Branquinho, C., Brown, D.H., Catarino, F., 1997. The cellular location of Cu in lichens and its effects on membrane integrity and chlorophyll fluorescence. Environ. Exp. Bot. 38, 165–179.
Brown, D.H., Beckett, R.P., 1983. Differential sensitivity of lichens to heavy metals. Ann. Bot. 52, 51–58.
Chettri, M.K., Cook, C.M., Vardaka, E., Sawidis, T., Lanaras, T., 1998. The effect of Cu, Zn and Pb on the chlorophyll content of the lichens *Cladonia convoluta* and *Cladonia rangiformis*. Environ. Exp. Bot. 39, 1–10.
Chisholm, J.E., Jones, G.C., Purvis, O.W., 1987. Hydrated copper oxalate, moolooite, in lichens. Mineral. Mag. 51, 715–718.
Collins, C.R., Farrar, J.F., 1978. Structural resistance to mass transfer in the lichen *Xanthoria parietina*. New. Phytol. 81, 71–83.
Clark, A., 1990. Inference of haplotypes from PCR-amplified samples of diploid populations. Mol. Biol. Evol. 7, 111–122.
Doering, M., Piercey-Normore, M.D., 2009. Genetically divergent algae shape: an epiphytic lichen community on Jack Pine in Manitoba. Lichenologist 41, 1–12.
Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.
Friedl, T., 1987. Thallus development and phycobionts of the parasitic lichen *Diploschistes muscorum*. Lichenologist 19, 183–191.
Garty, J., 2001. Biomonitoring atmospheric heavy metals with lichens: theory and application. Crit. Rev. Plant Sci. 20, 309–371.
Garty, Y., Karary, Y., Harel, Y., 1992. Effect of low pH, heavy metals and anions on chlorophyll degradation in the lichen *Ramalina duriaei* (De Not.) Bagl. Environ. Exp. Bot. 32, 229–241.
Gushina, I.A., Harwood, J.L., 2006. Lead and Cu effects on lipid metabolism in cultured lichen photobionts with different phosphorus status. Phytochemistry 67, 1731–1739.
Guzow-Krzemińska, B., 2006. Photobiont flexibility in the lichen *Protoparmeliopsis muralis* as revealed by ITS rDNA analyses. Lichenologist 38, 469–476.
Hauck, M., Helms, G., Friedl, T., 2007. Photobiont selectivity in the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*. Lichenologist 39, 195–204.
Helms, G., 2003. Taxonomy and symbiosis in associations of Physciaceae and *Trebouxia*. Ph.D. Thesis, University of Gottingen, Germany.
Helms, G., Friedl, T., Rambold, G., Mayrhofer, H., 2001. Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. Lichenologist 33, 73–86.
Honegger, R., 1992. Lichens, mycobiont–photobiont relationships. In: Reiser, W. (Ed.), Algae and Symbioses: Plants, Animals, Fungi, Viruses, Interactions Explored. BioPress Ltd., Bristol, UK, pp. 255–275.
Jahns, H.M., 1988. The establishment, individuality and growth of lichen thalli. Bot. J. Linn. Soc. 96, 21–29.
Kovář, P., 2004. Industrial deposits of abandoned sedimentation basins – technology of the origin and vegetation. In: Kovář, P. (Ed.), Natural Recovery of Human-Made Deposits in Landscape (Biotic Interactions and Ore/Ash–Slag Artificial Ecosystems). Academia, Praha, pp. 15–29.
Kroken, S., Taylor, J.W., 2000. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. Bryologist 103, 645–660.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.
Loppi, S., Pirintsos, S.A., De Dominicis, V., 1999. Soil contribution to the elemental composition of epiphytic lichens (Tuscany, central Italy). Environ. Monit. Assess. 58, 121–131.
Mukhtar, A., Garty, J., Galun, M., 1994. Does the lichen alga Trebouxia occur free-living in nature – further immunological evidence. Symbiosis 17, 247–253.
Nash, T.H., 1989. Metal tolerance in lichens. In: Shaw, A.J. (Ed.), Heavy Metal Tolerance in Plants: Evolutionary Aspects. CRC Press, Boca Raton, pp. 119–131.
Nelsen, M.P., Gargas, A., 2009. Symbiont flexibility in *Thamnolia vermicularis* (Pertusariales, Icmadophilaceae). Bryologist, 112, 404-417.
Nylander, J.A.A., 2004. MrModeltest v2. program distributed by the author. Evolutionary Biology Centre, Uppsala University.
Ohmura, Y., Kawachi, M., Kasai, F., Watanabe, M., 2006. Genetic combinations of symbionts in a vegetatively reproducing lichen, *Parmotrema tinctorum*, based on ITS rDNA sequences. Bryologist 109, 43–59.
Opanowicz, M., Grube, M., 2004. Photobiont genetic variation of *Flavocetraria nivalis*. Lichenologist 36, 125–131.
Orange, A., James, P.W., White, F.J., 2001. Microchemical methods for the identification of lichens. British Lichen Society, London.
Palice, Z., Soldán, Z., 2004. Lichen and bryophyte species diversity on toxic substrates in the abandoned sedimentation basins of Chvaletice and Bukovina. In: Kovář, P. (Ed.), Natural Recovery of Human-Made Deposits in Landscape (Biotic Interactions and Ore/Ash–Slag Artificial Ecosystems). Academia, Praha, pp. 200–221.
Paul, A., Hauck, M., 2006. Effects of manganese on chlorophyll fluorescence in epiphytic cyano- and chlorolichens. Flora 201, 451–460.
Pawlik-Skowrońska, B., di Toppi, L.S., Favali, M.A., Fossati, F., Pirszel, J., Skowroński, T., 2002. Lichens respond to heavy metals by phytochelatin synthesis. New Phytol. 156, 95–102.
Peksa, O., 2009. Species composition and diversity of lichens on anthropogenic substrata. In: Neustupa, J. (Ed.), The biological soil crusts in Central European ecosystems, with special reference to taxonomic structure and ecology of the surface crusts at Czech ore-waste and ash-slag sedimentation industrial basins. Novit. Bot. Univ. Carol. 19, Prague, pp. 38–40.
Piercey-Normore, M.D., 2004. Selection of algal genotypes by three species of lichen fungi in the genus *Cladonia*. Can. J. Bot. 82, 947–961.
Piercey-Normore, M.D., 2006. The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. New Phytol. 169, 331–344.
Piercey-Normore, M.D., DePriest, P.T., 2001. Algal-switching among lichen lichen symbioses. Am. J. Bot. 88, 1490–1498.
Purvis, O.W., 1984. The occurrence of copper oxalate in lichens growing on copper sulphide-bearing rocks in Scandinavia. Lichenologist 16, 197–204.
Purvis, O.W., Halls, C., 1996. A review of lichens in metal-enriched environments. Lichenologist 28, 571–601.
Romeike, J., Friedl, T., Helms, G., Ott, S., 2002. Genetic diversity of algal and fungal partners in four species of *Umbilicaria* (Lichenized Ascomycetes) along a transect of the Antarctic Peninsula. Mol. Biol. Evol. 19, 1209–1217.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Sanità di Toppi, L., Musetti, R., Marabottini, R., Corradi, M.G., Vattuone, Z., Favali, M.A., Badiani, M., 2004. Responses of *Xanthoria parietina* thalli to environmentally relevant concentrations of hexavalent chromium. Funct. Plant Biol. 31, 329–338.
Sanità di Toppi, L., Musetti, R., Vattuone, Z., Pawlik-Skowrońska, B., Fossati, F., Bertoli, L., Badiani, M., Favali, M.A., 2005. Cadmium distribution and effects on ultrastructure and chlorophyll status in photobionts and mycobionts of *Xanthoria parietina*. Microsc. Res. Tech. 66, 229–238.
Seaward, M.R.D, 1973. Lichen ecology of the scunthorpe heathlands. I. mineral accumulation. Lichenologist 5, 423–433.
Schuster, G., Ott, S., Jahns, H.M., 1985. Artificial cultures of lichens in the natural environment. Lichenologist 17, 247–253.
Swofford, D,L., 2003. In: PAUP*. Phylogenetics analysis using parsimony (and other methods). Version 4.. Sinauer Associates, Sunderland, Massachusetts.
Tarhanen, S., 1998. Ultrastructural responses of the lichen Bryoria fuscescens to stimulated acid rain and heavy metal deposition. Ann. Bot. 82, 735-746.

Verbruggen, H., Theriot, E.C., 2008. Building trees of algae: some advances in phylogenetic and evolutionary analysis. Eur. J. Phycol. 43, 229–252.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Yahr, R., Vilgalys, R., DePriest, P.T., 2004. Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. Mol. Ecol. 13, 3367–3378.

Yahr, R., Vilgalys, R., DePriest, P.T., 2006. Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. New Phytol. 171, 847–860.