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The study of photobionts in the lichenized genus *Lepraria*

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Contents

I hereby declare that I made this thesis independently, using the listed references, or in the cooperation with other authors of the papers (for my contribution to particular papers see chapter 8 Author´s contribution).

I did submit neither the thesis nor its any part to acquire any other academic title.

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Ondřej Peksa

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2 Abstract

My study is focused on green algal photobionts – lichenized algae from the genera *Asterochloris* and *Trebouxia* (the last was studied in a lesser extent). The principal objectives of the thesis can be characterized as follows: 1) to investigate the chloroplast morphology in lichenized algae *Asterochloris* and *Trebouxia*; 2) to study the photobiont diversity in lichen community growing in heavy metal polluted habitats; 3) to inventorize the photobionts in selected lichen taxa (*Lepraria*, *Stereocaulon*) in order to reveal their ecological requirements.

Using confocal microscopy, significant differences in the chloroplast structure of lichenized and cultured (free-living) algae were detected. Moreover, algae exhibited gradual changes in chloroplast shape and structure during successive phases of cultivation – from the relatively simple lichenized form to the complex lobate chloroplast with several ontogenetic (morphological) stages observable in mature axenic cultures.

Several *Asterochloris* lineages and one *Trebouxia* species were detected in terricolous lichen community from metal-polluted habitats using phylogenetic analysis of algal ITS sequences. All these lineages represented photobionts with broad ecological amplitude and worldwide distribution. In several taxa of pioneer lichens, low specificity as well as selectivity of lichen-forming fungi to their photobionts was revealed. They associated with several algal lineages (even within one thallus). Therefore, they can serve as an important photobiont transferring system.

Using phylogenetic analysis based on the concatenated set of ITS rDNA and actin type I locus sequences, 13 well resolved clades of *Asterochloris* were found to be associated with *Lepraria* and *Stereocaulon* species investigated. Rather low specificity and intensive switching of photobionts was revealed in asexual *Lepraria*. Photobionts from particular algal clades were found in taxonomically different but ecologically similar lichens. The rain and sun exposure was the most significant environmental factor, clearly distinguishing the *Asterochloris* lineages. Moreover, two photobiont lineages were obviously differentiated based on their substrate and climatic preferences. These environmental preferences of photobionts control the ecology of lichens and lead to the existence of specific lichen guilds.

During the whole study, almost 240 new photobiont sequences from 154 lichen specimens were produced. From a total of 39 species of lichen-forming fungi, eight species were investigated that have not been analysed for their photobionts to date. Many new lineages of lichenized algae were revealed.

Abstrakt (in Czech)

Předkládaná disertační práce je zaměřena na lichenizované zelené řasy rodu *Asterochloris* a *Trebouxia* (zástupci druhého rodu byli studováni v menší míře). Dílčí cíle práce byly následující: 1) studovat morfologii chloroplastu lichenizovaných řas r. *Asterochloris* a *Trebouxia*; 2) studovat diverzitu fotobiontů v lišejnících rostoucích na stanovištích s vysokým obsahem těžkých kovů; 3) studovat fotobionty lišejníků r. *Lepraria* a *Stereocaulon* s cílem odhalit jejich ekologické preference.

S použitím konfokální mikroskopie jsem odhalil zásadní rozdíly ve struktuře chloroplastu lichenizovaných a volně žijících (kultivovaných) fotobiontů. Během samovolného uvolňování řas z pletiv lišejníku jsem navíc pozoroval postupné změny chloroplastu. Ten se měnil z jednoduché kompaktní formy přítomné v lichenizované řase do komplexní laločnaté formy s různými ontogenetickými stádii pozorované v řase volně žijící.

S použitím fylogenetické analýzy sekvencí ITS rDNA se podařilo v terikolních lišejnících rostoucích na lokalitách znečištěných těžkými kovy rozlišit několik linií řas r. *Asterochloris* a jednu linie r. *Trebouxia*. Všechny tyto linie representovaly fotobionty s širokou ekologickou amplitudou a rozsáhlým areálem. Řada pionýrských lišejníků vykazovala malou specificitu i selektivitu mykobiontů vůči fotobiontům – houby se sdružovaly s několika liniemi řas, v některých případech i v rámci jediné stélky. Díky této nízké specificitě a zároveň intenzivnímu vegetativnímu šíření mohou pionýrské lišejníky sloužit jako významný systém pro přenos a šíření fotobiontů.

S použitím fylogenetické analýzy založené na sekvencích ITS rDNA a genu pro aktin bylo v lišejnících rodu *Lepraria* a *Stereocaulon* odhaleno 13 linií fotobiontů r. *Asterochloris*. Druhy rodu *Lepraria* (zcela sterilní) vykazovaly spíše malou specificitu vůči fotobiontům a jejich intenzivní výměnu. Jednotlivé linie fotobiontů byly nalezeny v taxonomicky různých, ale ekologicky podobných lišejnících. Nejsilnějším faktorem ovlivňujícím přítomnost toho kterého fotobionta ve stélce se ukázala být exponovanost vůči dešti a slunci. Navíc byly nalezeny dvě linie řas, jejichž výskyt se řídil typem substrátu a nadmořskou výškou. Tyto ekologické preference fotobiontů ovlivňují ekologii lišejníků jako takových a vedou k existenci specifických lišejníkových gild (společenství).

Během celé práce jsem získal téměř 240 nových sekvencí fotobiontů ze 154 položek lišejníků. Osm druhů lišejníků (z 39 analyzovaných) nebylo předtím z hlediska fotobiontů vůbec zkoumáno. Podařilo se mi odhalit několik nových linií lichenizovaných řas.

3 General introduction

The lichen as a complex symbiotic entity is typically composed of a lichenized fungus – the mycobiont – and an alga or cyanobacteria – the **photobiont**. The mycobiont is mostly the dominant constituent of the lichen thallus, it forms obvious vegetative as well as reproductive structures such as rhizines, fruit bodies, pycnids, etc. On the other hand, the lichenized algae and cyanobacteria are usually hidden within fungal tissues. This fact probably evoked the traditional view of lichen as a single plant with a single name (the current nomenclature refers only to the lichen-forming fungus). The dual nature of lichens was discovered in 1867 by Simon Schwendener who recognized the photobionts as distinct symbiotic entities.

Although the photobiont represents mainly an inconspicuous part of a lichen, it is by no means just a „supplement“ to the mycobiont. The self-sufficiency of the lichen is actually caused by the autotrophic partner that nourishes the fungus by its photosynthetic products. Thus, the photobiont constitutes an essential part of a lichen.

Unsuprisingly, our knowledge of the photobionts is far from that of the mycobionts. Even after the investigation of photobionts became easily practicable and precise, thanks to the advent of molecular techniques, it represents only a small portion of lichen research (at the last two international lichenological meetings IAL 5 and IAL 6, only 7 % of the presented works were focused on photobionts; Randlane & Saag 2004, Nash III & Seaward 2008). However, more detailed investigation of photobionts is necessary to obtain answers about the formation and functioning of lichens, a very successful and important group of symbiotic organisms.

This study provides a contribution to the knowledge of symbiotic lichenized algae as well as the relationships between the photobionts and their heterothrophic fungal hosts.

The research of photobionts has numerous aspects. In the following text, I tried to briefly outline some important issues – the identity, taxonomy, biodiversity and ecology of photobionts as well as the specificity and selectivity of both symbiotic partners.

3.1 Photobiont identity

Since Schwendener's discovery of the dual nature of lichens (Schwendener 1867), the factual identity of the green symbionts (photobionts) in lichen thalli has been questioned by lichenologists. However, the exploration of photobionts turned out to be far from easy. The simple identification *in situ* (within a lichen thallus) was found to be impossible because the morphology of the lichenized alga as well as cyanobacteria is usually altered by the influence of the mycobiont (cf. Bubrick 1988, Tschermak-Woess 1988). Typically, a remarkable reduction of cell size is found in the majority of eukaryotic bionts. Filamentous algae and cyanobacteria are generally reduced to unicellular forms. Both sexual and asexual reproductive strategies of algae and the cell division of cyanobacteria are usually modified. At the subcellular level, modifications of the size, structure, as well as the number and distribution of some organelles and cellular structures were detected.

Thus, the photobionts must be isolated from lichen thalli to the axenic culturs. Only such „free-living" algae can be identified according to the traditional methods based on morphological characters. Ahmadjian (1967) published the first complete guide for isolation, culturing and identification of lichen photobionts. The same author created the summarizing determination key to the morphological traits of common genera of lichenized algae and cyanobacteria (Ahmadjian 1958; later modifications by Poelt 1969, Gärnter 1992, Ettl & Gärtner 1995 etc.). Taxonomy at the species level was elaborated only in some genera, especially in the genus *Trebouxia* (including *Asterochloris*)[1]. The applicable differentiation of individual species was based mainly on the cell shape, type of sporogenesis, morphology of the chloroplast (Ahmadjian 1960, Gärtner 1985) and ultrastructure of the pyrenoid (Friedl 1989a, b). A number of keys to other genera containing green-algal photobionts (*Myrmecia*, *Dictyochloropsis*, *Elliptochloris* etc.) were included in the Syllabus der Boden–, Luft– und Flechtenalgen (Ettl & Gärtner 1995). However, although based on cultured strains, the morphological determination may become very difficult and unreliable in certain cases. Several important morphological characters exhibit high plasticity, the differences in culturing methods may lead to various phenotypic expression, etc. Moreover, there exist many undescribed species (e.g. Lücking et al. 2009, Nelsen & Gargas 2008) which can not be correctly classified using the current determination keys. Therefore, the application of

[1] *Asterochloris* has been included within *Trebouxia* for a long time (see e.g. chapter „*Trebouxia – Asterochloris*, one or two genera?" in Helms 2003, p. 41–42; or *Paper 5* of this thesis)

compound approaches combining at least morphological and molecular data is necessary for reliable identification of the lichen algae and cyanobacteria (Škaloud 2008).

In total, about 100 species in 45 genera of algae and cyanobacteria have been reported from lichens to date (Friedl & Büdel 2008, Tschermak-Woess 1988; see Table 1)[2].

The most frequently reported photobionts belong to the genera *Nostoc*, *Trebouxia*, *Asterochloris* and *Trentepohlia* (Friedl & Büdel 2008). *Trebouxia* (incl. *Asterochloris*) is mentioned as the most common lichenized alga occuring in more than a half of all known lichens (Ahmadjian 1993). Together with another Trebouxiophyceae (e.g. *Coccomyxa*, *Dictyochloropsis*, *Elliptochloris*), it predominates in all families of lichen fungi except those from Peltigerales and Ostropales s.l. (cf. Miadlikovska et al. 2006).

Trebouxiophyceae (and Ulvophyceae) seem to be the exclusive „providers" of lichen photobionts (Lewis & McCourt 2004). Moreover, in addition to lichens, trebouxiophyte algae also participate in other symbiotic interactions – they occure endophytically in the gametophytic tissues of *Ginkgo biloba* (Tremouillaux-Guiller et al. 2002); the water species live as endosymbionts of fresh water ciliates (Summerer et al. 2008), *Hydra viridissima* (Huss et al. 1993/94) and sea anemones (Letsch et al. 2009, Lewis & Muller-Parker 2004). The members of Trebouxiophyceae seem to be well predisposed to the symbiotic lifestyle. On the other hand, in Chlorophyceae, another very abundant class of green algae, the proportion of taxa participating in symbiosis is surprisingly small (e.g. Friedl & Büdel 2008, Pröschold et al. 2010).

3.2 Biodiversity of photobionts

It is rather difficult to estimate true biodiversity of photobionts due to the lack of information. About 700 genera of lichen-forming fungi were described (cf. Theler & Wedin 2008), but only about 20% of them include the members analysed for their photobionts (cf. Tschermak-Woess 1988 and further current studies). Actually, not a single genus has been properly and thoroughfully investigated for its algal symbionts. One of the most intensively investigated genus is *Cladonia*, in which 80 from the estimated 450 species have been examined for their photobionts to date (see Appendix 1 – List of lichen fungi associated with *Asterochloris*).

[2] However, these data are based on investigation of only several percent of lichen taxa in which the photobiont has ever been identified at species or generic level. On the other hand, a number of recognized photobiont taxa belong to „uncertain" species (e.g. Ettl & Gärtner 1995, p. 449). Thus, the mentioned numbers of photobiont taxa are very incomplete.

Table 1. The list of genera of cyanobacteria and algae detected as photobionts (based on Büdel 1992, Darienko et al. 2010, Ettl & Gärtner 1995, Friedl & Büdel 2008, Gärtner 1992, Kohlmeyer et al. 2004, Lücking et al. 2009, Pérez-Ortega et al. 2010, Tschermak-Woess 1988). In addition to the genera listed, there exist some other problematic records of „photobionts" from lichens (see Gärtner 1992, p. 329). The affiliation of photobiont genera to orders and families follows current knowledge. However, there is no possibility to verify the accuracy of photobiont identification. Moreover, in many cases, the photobiont was incompletely determined, therefore, its affiliation to a higher taxon can not be accurate (especially in problematic taxa like *Chlorella* or *Gloeocystis*).

Cyanobacteria		**Eukaryotic algae**	
Anabaena	Nostocales	***Asterochloris***	Trebouxiophyceae
Aphanocapsa	(Synechococcales)	***Cephaleuros***	Ulvophyceae
Calothrix	Nostocales	***Coccobotrys***	Chlorophyceae
Cyanosarcina	Chroococcales	***Coccomyxa***	Trebouxiophyceae
Dichothrix	Nostocales	***Desmococcus***	Chlorophyceae
Entophysalis	Chroococcales	***Dictyochloropsis***	Trebouxiophyceae
Gloeocapsa	Chroococcales	***Dilabifilum***	Ulvophyceae
Hormathonema	Chroococcales	***Diplosphaera***	Chlorophyceae
Hyella	Chroococcales	***Elliptochloris***	Trebouxiophyceae
Hyphomorpha	Stigonematales	***Gloeocystis***	Chlorophyceae
Chroococcidiopsis	Chroococcales	***Heterococcus***	Xanthophyceae
Chroococcus	Chroococcales	***Chlorella***	Trebouxiophyceae
Microcystis	Chroococcales	***Chloroidium***	Trebouxiophyceae
Myxosarcina	Chroococcales	***Chlorosarcinopsis***	Chlorophyceae
Nostoc	Nostocales	***Leptosira***	Trebouxiophyceae
Rhizonema	Nostocales	***Myrmecia***	Trebouxiophyceae
Scytonema	Nostocales	***Nannochloris***	Trebouxiophyceae
Stigonema	Nostocales	***Pelvetia****	Phaeophyceae
Tolypothrix	Nostocales	***Petroderma***	Fucophyceae
		Phycopeltis	Ulvophyceae
		Prasiola*	Trebouxiophyceae
		Protococcus**	Trebouxiophyceae
		Pseudochlorella	Trebouxiophyceae
		Stichococcus	Trebouxiophyceae
		Trebouxia	Trebouxiophyceae
		Trentepohlia	Ulvophyceae
		Trochiscia	Chlorophyceae

* photobiont of marine "borderline lichen" *Collemopsidium pelvetiae*
** photobiont of marine "borderline lichen" *Mastodia tessellata*
*** synonimized with *Apatococcus*

Knowledge of the photobiont diversity within individual fungal species is slightly better, some of them have been relatively deeply examined (e.g. Guzow-Krzemińska 2006, Nelsen & Gargas 2009, Nyati 2007, Piercey-Normore 2006, Tschermak-Woess 1995).

Nevertheless, we can presume that the biodiversity of lichenized algae and cyanobacteria is much higher than the 100 species currently known. The molecular studies suggest the existence of many undescribed species. For example, Helms (2003) analysed photobionts in

some taxa of the family Physciaceae. He detected 31 algal subclades within four clades based on analysis of ITS sequences, but only 12 of them belonged to described *Trebouxia* species (Fig. 1). So, he revealed 19 lineages potentially representing new species (to date, only 19 other species of *Trebouxia* were formally described!). Moreover, a number of lineages probably representing further new species have been discovered in *Trebouxia* (Blaha et al. 2006, Kroken & Taylor 2000, Romeike et al. 2002) as well as in the related genus *Asterochloris* (Beiggi & Piercey-Normore 2007, Cordeiro et al. 2005, Piercey-Normore & DePriest 2001, Yahr et al. 2006).[3] A novel, previously unrecognized, exclusively lichenized lineage of filamentous cyanobacteria named *Rhizonema* was recently reported from tropical lichens based on analysis of 16S rRNA sequences (Lücking et al. 2009). Many new lineages of *Nostoc* were detected during the research of Peltigerales (e.g. Myllys et al. 2007) and Collemataceae (Otálora et al. 2010). Therefore, we can probably anticipate many new taxa of lichenized algae as well as cyanobacteria during the investigations of photobionts in other lichen groups.

On the other hand, we can consequently suppose that the number of photobionts will be lower than that of mycobionts. The majority of photobionts have been found associated with several mycobiont species or even genera (they are shared by them). For example, cyanobacterial genus *Rhizonema* associates with 3 fungal genera (Lücking et al. 2009) (Fig. 2), *Coccomyxa subellipsoidea* is shared by six species of basidiomycete *Omphalina* (Zoller & Lutzoni 2003), *Trebouxia hypogymniae* ined. was detected in eight fungal genera including many species (Hauck et al. 2007, Piercey-Normore 2009), *Asterochloris glomerata* associates with almost 50 fungal species from several genera (e.g. Piercey-Normore & DePriest 2001, Cordeiro et al. 2005, Yahr et al. 2004).

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[3] *Asterochloris* and *Trebouxia* are the most abundant as well as the most investigated lichen-forming algae. Seven species of *Asterochloris* and 19 species of *Trebouxia* were formally described. However, the current morphospecies concepts of these genera were found not to be suitable to accurately account for their genetic diversity. Some morphospecies were revealed to be conspecific based on their ITS or actin sequences, consequently, many undescribed lineages in both genera have been detected by molecular studies. However, these new lineages are not formally described, the sequences remain their only differential characters. Therefore, the identification of photobionts is usually constricted to the assignment to a particular clade. The researchers do not seem to be interested in delimitation and description of new species. Unfortunatelly, the researchers of photobionts do not often keep a continuity in clade labelling, as known for example from the studies of coral zooxanthelae *Symbiodinium* (from Rowan & Knowlton 1995, all the researchers keep a clade naming A, B, C, D, E). This fact makes the orientation in the photobiont diversity very difficult.

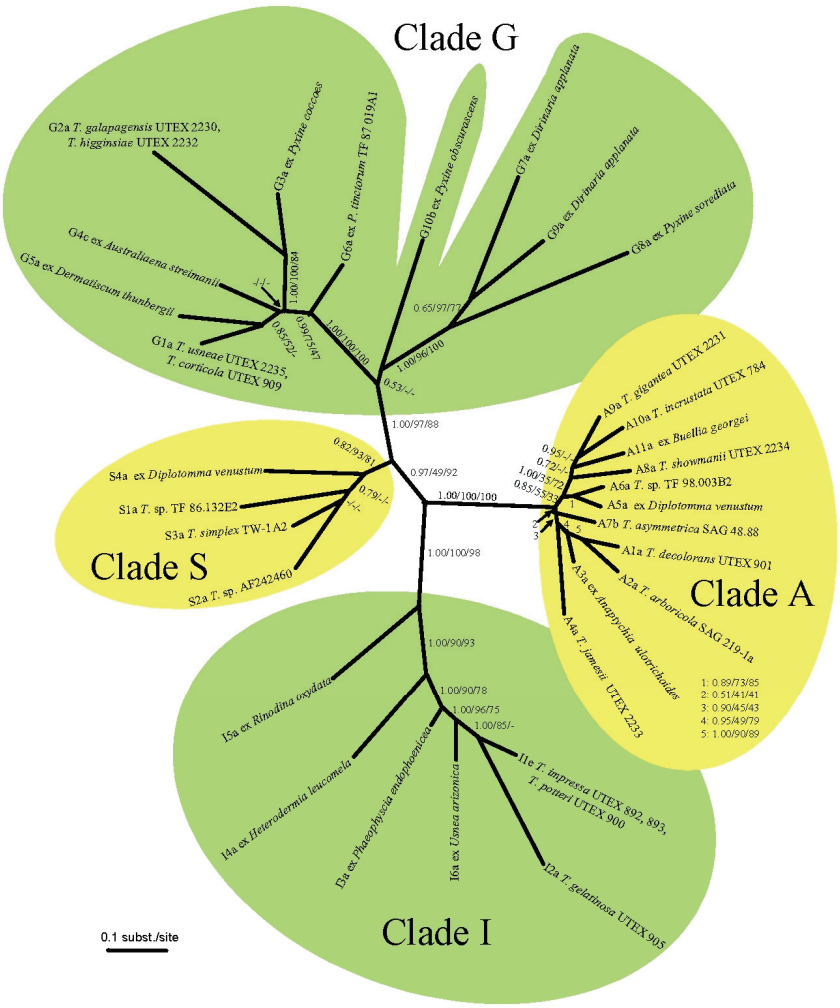

Fig. 1. *The molecular studies revealed the existence of many undescribed photobiont species*. Unrooted maximum likelihood phylogeny of photobionts (*Trebouxia*) associated with the family Physciaceae. From the 31 subclades detected, only 12 belonged to the described *Trebouxia* species (Helms 2003, p. 55, fig. 4.3).

These differences in diversity of the lichen-forming fungi and algae could have arisen due to different rates of evolution in both groups. In symbiotic systems, where one partner lives extracellularly inside the other one (the majority of lichens comply with this definition), the inhabitant should have lower rate of genetic change (Law & Lewis 1983), because it lives under more stable conditions provided by the exhabitant. In contrast, the exhabitant is exposed to a higher pressure of abiotic factors as well as competition, leading to its adaptive (genetic) changes. This hypothesis was examined in *Cladonia* lichens (Piercey-Normore & DePriest 2001) and especially in *Omphalina* associated with the green alga *Coccomyxa*

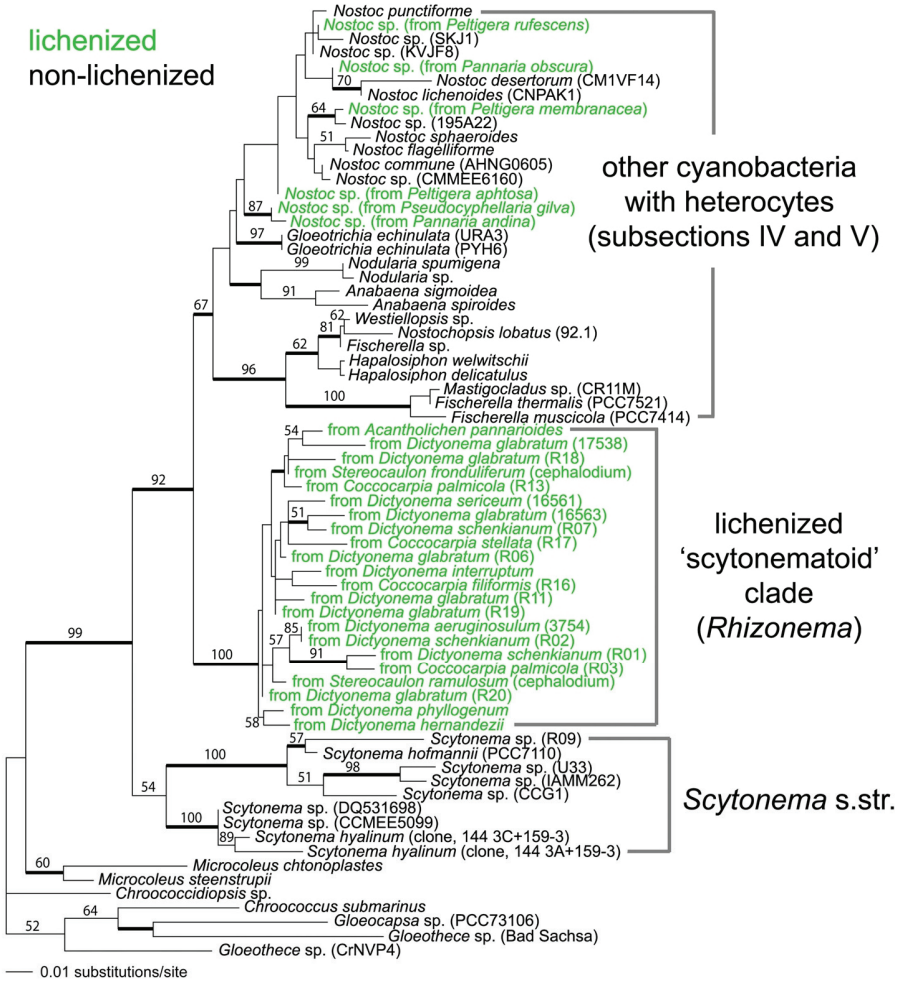

Fig. 2. *Most of the photobionts are shared by several lichen-forming fungi*. Phylogenetic relationships of lichenized scytonematoid photobionts to other lichenized and free-living cyanobacteria, inferred from 16S rRNA sequences using maximum likelihood (Lücking et al. 2009, p. 1413, fig. 1). The cyanobacterial genus *Rhizonema* associates with many species within 3 fungal genera.

(Zoller & Lutzoni 2003). These authors found that the substitution rates in the ITS region of *Omphalina* pairs were much higher than the rates in the corresponding pairs of *Coccomyxa*. They speculated that „it is not clear at this point if the low substitution rates observed in symbiotic algae are a direct result of the lichenization, or if these species were favored by the fungi because they already had the predisposition needed for a successful and stable transition to a lichenized state (low mutation rates, mostly asexual reproduction, etc.)“. Nevertheless, if the differences in the rates of genetic change between photobionts and mycobionts are common in lichens (regardless of their backround), we can assume also different diversities in both groups.

3.3 Biogeography of photobionts

An interesting perspective on diversity of lichen photobionts is provided by their biogeography. Both cyanobacteria and algae are microscopic organisms. Moreover, the vegetative propagules of lichens containing both symbionts (soredia, isidia) are mostly not much larger than particular algal cells (20–50 μm, Büdel & Scheidegger 2008) and they are capable of being dispersed over large distances as well (Bailey 1976). The well-known theory of ubiquitous dispersal of microbial species (Finlay & Clarke 1999) presumed that most organisms smaller then ca 1 mm should occur worldwide (in a niche-based context only). According to this theory, the global species richness of photobionts should be rather low, because the unrestricted dispersal results in low rates of allopatric speciation (Fenchel 1993). Actually, some photobiont lineages are apparently widely distributed (e.g. *Trebouxia jamesii* agg., some *Nostoc* lineages). However, other were reported only from specific regions suggesting the existence of some biogeographical patterns of their distribution. Moreover, there are number of endemic taxa among lichens (23% in New Zealand, Galloway 2007; 50% in continental Antarctica, Øvstedal & Lewis Smith 2001), whose endemism could be explained by narrow distribution (local adaptation) of mycobiont or photobiont.

3.4 Ecology of photobionts

More then 13 500 lichen (fungal) species have been described (Hawksworth & Hill 1984), occuring in almost all terrestrial ecosystems (only several lichen taxa live in water conditions). Lichens grow in various habitats, on a wide range of substrates. Generally, many taxa exhibit specific requirements to the climate (tropical/temperate/arctic species), substrate (epiphytes/terricolous/saxicolous; basiphilous/acidophilous), etc.

At the first sight, the mycobiont seems to be the main determinant of the ecological requirements of a lichen. It plays a role of the exhabitant which is exposed to the direct influence of external conditions, while the photobiont is usually confined to a thin layer inside the thallus, protected from above as well as below by fungal tissues. Actually, the mycobiont can shelter its partner against the harmful UV radiation, it can partially regulate the water content and distribution within the thallus (Honegger 2006, 2009), or it can protect the photobiont against the direct influence of substrate pH (Mollenhauer 1997). Nevertheless, the possibilities of lichen-forming fungi to protect their photobionts are necessarily limited due to poikilohydric nature of lichens. The extreme conditions in cold Antarctica or in hot deserts

likely test the endurance of both partners, not only the mycobiont. Furthermore, the ecology of the algae or cyanobacteria has its own history, preceding the lichenization event. Some photobionts are still recruited from free-living forms (*Nostoc*; Wirtz et al. 2003), other were found only in form of small free-living colonies whose survival is probably strongly dependent on suitable local conditions (*Trebouxia*; Tschermak-Woess 1978, Bubrick et al. 1984, Nakano 1985, Mukhtar et al. 1994, Sanders 2005)[4].

Although they can adapt to the changing sun irradiance through the regulation of photosynthetic pigments concentrations (Czeczuga et al. 2010, Paoli et al. 2010), each species of alga or cyanobacterium probably differs in its optimum for photosynthetic activity. An unsuitable light regime in the habitat may cause that the photobiont can not nourish the whole system of lichen effectively. Beside this, the metabolic activity of the photobiont is affected by other factors such as water conditions, temperature, nutrients abundance, etc. Therefore, it is an indisputable advantage for a mycobiont to find a locally adapted, well prospering photobiont. Only a good viability of the photobiont can ensure a good fitness of the fungus as well as of the whole lichen under specific local conditions. This fact probably leads to the switching of photobionts, documented in both sexually and vegetativelly reproducing lichen-forming fungi (e.g. Blaha et al. 2006, Guzow-Krzemińska 2006, Muggia et al. 2008, Nelsen & Gargas 2008, 2009, Romeike et al. 2002, Yahr et al. 2006).

Many findings refer to the existence of photobionts adapted to a specific habitat or geographical region with different environmental conditions, in both cyanolichens and green algal lichens.

Cyanobacterial photobionts

Limestone hosts a significantly higher proportion of cyanolichens in comparison with the most of other rock types (Pentecost & Whitton 2000). Cyanolichens have also been found as very vulnerable to the decreased pH levels caused by acid rains (Ahmadjian 1993). Actually, positive relationship to the basic (high-pH) substrates is the main characteristic of cyanobacteria. Acidic substrates are one of the stressfull environments for them. Most cyanobacteria are normally absent at pH values below 4.0 or 5.0 (eukaryotic algae, however, flourish under these conditions; Fogg et al. 1973). Therefore, lichenized cyanobacteria probably underlie the prevailing occurence of cyanolichens in basic environments.

[4] There exists a „dilemma“ of free-living *Trebouxia*. It was discussed especially by Ahmadjian (1967, 1988).

In addition, the differences in ecological requirements of the cyanobacterial photobionts have been suggested to explain the existence of cyanolichen guilds (communities of lichens growing in the same habitat, sharing the same photobiont) in old-growth forests. Rikkinen et al. (2002) found the Nostoc strains from epiphytic lichens distinctly separated from those associated with terricolous ones (*Nephroma* and *Peltigera* guild, respectively). This separation was probably caused by their adaptation to different water and nutritional conditions on the ground and on tree trunks.

Green algal photobionts

Algae from the green algal order Trentepohliales (Ulvophyceae) are wide-spread in regions with (sub-)tropical climate. Although they are also present in temperate regions, they are most abundant and diverse in the tropics (López-Bautista et al. 2006). Therefore, it is no wonder that lichens containing photobionts from Trentepohliales (*Trentepohlia*, *Phycopeltis*, *Cephaleuros*) are much more frequent in tropical and subtropical regions than in the temperate belt. In Western Europe, specific increase of lichens containing *Trentepohlia* as phycobiont has been recently observed. All these taxa have in common a southern distribution, and their shifting is probably caused by global warming (Aptroot & van Herk 2007). This case illustrates the distinct influence of photobiont ecology to the distribution of lichens.

A relevant role of substrate pH and climate has been detected in distribution of trebouxioid photobionts as well. During his approach on Physciaceae (Lecanorales), Helms (2003) revealed the *Trebouxia* phylogeny much closer correlated with environmental factors than the phylogeny of the mycobiont (Fig. 3). The subclades of *Trebouxia* found in the lichen taxa growing on calcareous rock formed a single lineage distinct from that of photobionts from the acidophilous lichens. Moreover, two other *Trebouxia* lineages (clade G, I4) were found to occur predominantly in the tropics. Consequently, the prevailing tropical character of these two clades was confirmed in two South-American studies (Cordeiro et al. 2005, Reis 2005). On the other hand, algae belonging to the *Trebouxia simplex* lineage seem to prefer cold climate. They have been detected in various lichens from different parts of the world, however, the majority of collections originated from montane regions (Blaha et al. 2006, Hauck et al. 2007, Muggia et al. 2008, Nelsen & Gargas 2009).

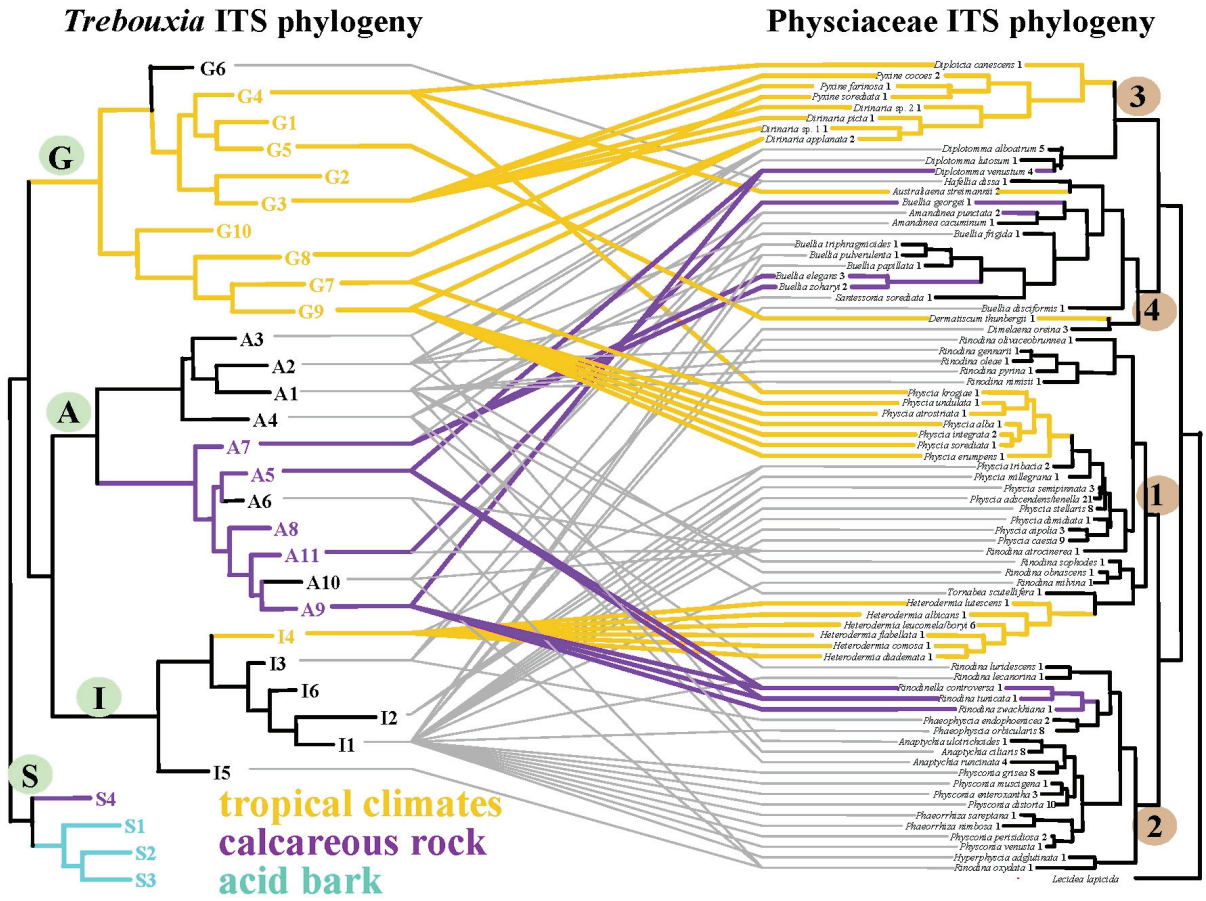

Fig. 3. *Some photobionts seem to be adapted to a distinct habitat or geographical region with different environmental conditions*. Correlations of *Trebouxia* and Physciaceae phylogenies with environmental parameters (Helms 2003, p. 93, fig. 5.6).

Some other *Trebouxia* lineages could be ecologically differentiated on the basis of their preferences to epiphytic and saxicolous lichens, or to trees and rocks, respectively. Helms (2003) observed certain *Trebouxia* ITS-variants to occur on rock or tree trunks with different frequencies. Similarly, Nyati (2007) reported a prevailing occurence of *Trebouxia arboricola* in saxicolous specimens of *Xanthoria*. On the other hand, *Trebouxia decolorans* was almost exclusively found in corticolous samples of these lichens.

Some negative evidence also suggests the existence of patterns in distribution and ecology of trebouxioid photobionts, i.e. some lineages are simply missing in distinct biotopes or regions (Nelsen & Gargas 2009, Yahr et al. 2006). If we consider the theory of ubiquitous dispersal valid, such cases could support the existence of environmental preferences in some photobionts (which seem to be more important for their distribution than the association with a particular mycobiont).

3.5 Specificity and selectivity

A lichen is an entity that originates and prospers based on symbiotic interaction between two (or more) partners. It has always been one of the most interesting question of lichenology – what are the rules of their partnership.

The specificity and the selectivity represent the major factors for determining the composition of particular symbiotic partnership (Yahr et al. 2004). The specificity means the number of partners with which an organism associates (Smith & Douglas 1987), the selectivity means the frequency of association among compatible partners (Rambold et al.1998). Specificity and selectivity do not have to be correlated. Low specificity of a host associating with several unrelated lineages of symbionts may be coincident with high selectivity, in terms of preferential association with only one of those lineages (Yahr et al. 2004).

Both terms are usually used only from the perspective of the fungal partner. Actually, the fungus acts as an active partner („a farmer searching for a suitable domesticant"). Moreover, taxonomy of lichen-forming fungi is usually clear in comparison with that of lichenized algae, therefore, one can easily define whose specificity he wants to examine. However, there is no reason not to use these terms from the perspective of the alga as well, at least in the case of specifity. Surely, an algal lineage (genus, species, clade) has also its specific range of compatible hosts.

The specificity of the mycobiont

The specificity can be described at different taxonomic levels. Many fungal genera or even families are apparently specific to a certain genera of algae or cyanobacteria (e.g. Parmeliaceae/*Parmelia* s.l. – *Trebouxia*, Cladoniaceae/*Cladonia* – *Asterochloris*, Collemataceae/*Collema*, *Leptogium* – *Nostoc*, Gyalectaceae/*Gyalecta* – *Trentepohlia*). However, individual species from these genera may range from high to low specificity for individual clades or genotypes of the photobionts.

Based on current knowledge (see below), the fungal species exhibiting rather low specificity (generalists) seem to be more frequent than those characterized by high specificity (specialists). Such result is not surprising because low specificity should provide an evolutionary advantage to the symbiotic fungi. A less specific mycobiont should be able to colonize various habitats occupied by different photobionts. For example, two really

widespread and ubiquitous lichens *Lecanora rupicola* and *Protoparmeliopsis muralis* have been reported as having very low specifity (Blaha et al. 2006, Guzow-Krzemińska 2006). Both species have associated with several unrelated *Trebouxia* lineages. This high flexibility in respect to the photobiont was probably the essence of their succesful colonization of different habitats and geographical regions. Similarly, another widespread lichens have been found to associate with more than one photobiont lineage (e.g. Muggia et al. 2008, Nelsen & Gargas 2009, Nyati 2007, Piercey-Normore 2006, Wornik & Grube 2010).

On the other hand, lichens with limited distribution, including endemic taxa, could theoretically exhibit only restricted range of compatible symbionts (high specificity). However, the investigation of two endemic species, *Umbilicaria antarctica* (Romeike et al. 2002) and *Cladonia subtenuis* (Yahr et al. 2006), did not confirm such hypothesis. Both species contained several unrelated lineages of *Trebouxia* and *Asterochloris*, respectively.

Nevertheless, some lichen-forming fungi have been detected as being associated with only one species or lineage of photobiont (e.g. Ohmura et al. 2006, Hauck et al. 2007, Opanowicz & Grube 2004, Otálora et al. 2010, Yahr et al. 2004, Werth & Sork 2010). However, in all these studies, lichen samples were collected in quite narrow areas (a single country, single mountain belt or single bay). A broader exploration of larger area and/or of more habitat types could provide the findings of other associated photobionts (as some authors noted themselves – Werth & Sork 2010, Yahr et al. 2004). A good example represents a study of Nelsen & Gargas (2009) who examined the genetic structure of algal partners in *Thamnolia vermicularis*, the widely distributed montane lichen. If the authors limited their sampling only to Europe and North America, they would conclude that this species is highly specific to only one lineage of *Trebouxia*. However, in specimens from China and Costa Rica they found three additional *Trebouxia* clades (Fig. 4), suggesting that in fact, *T. vermicularis* is rather less specific (although locally selective, see below).

In Collemataceae, two species have been reported as extremely specific to a distinct *Nostoc* clade although sampled in different continents – Europe and North America (Otálora et al. 2010). However, the species investigated (*Collema flaccidum*, *Leptogium saturninum*) had much wider geographic distributions. Thus, additional sampling in other regions (Asia, Australia, South America) could reveal other associated *Nostoc* lineages.

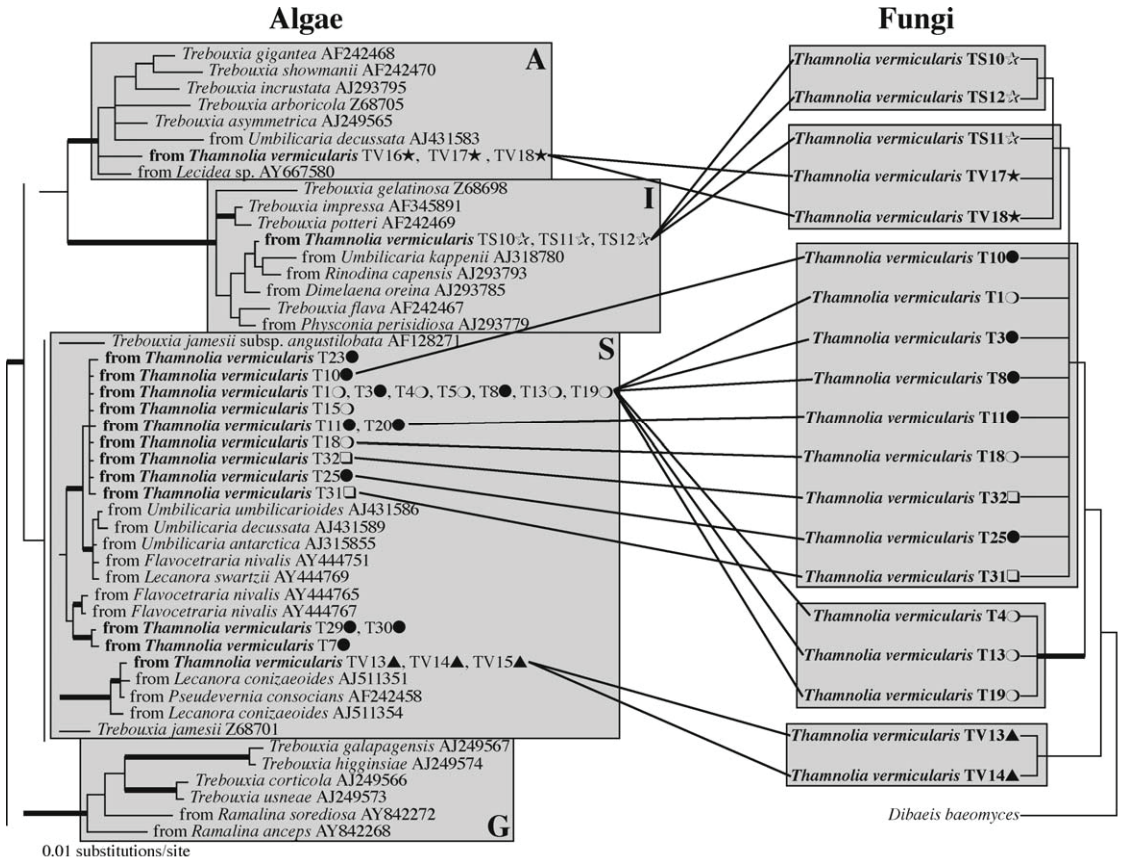

Fig. 4. *An exploration of large area or of several habitat types could change the notion of specificity in lichen association – in different geographical areas the fungus usually cooperates with another photobiont*. The study of genetic structure of algal partners in *Thamnolia vermicularis* (Nelsen & Gargas 2009, p. 410, fig. 1 – majority-rule consensus trees from Bayesian analyses of algal and fungal datasets; geographic origin: circle – USA; square – Norway; star – China; triangle – Costa Rica; filled and empty symbols distinguish the chemistry of mycobionts).

The selectivity of the mycobiont

Last examples of highly specific fungal species suggested that these taxa are locally selective towards distinct photobiont lineages rather than having a narrow range of compatible partners. Such selectivity could be evoked by combination of several factors: specificity of the mycobiont, local conditions, reproduction mode of a fungus and surely some random events (Yahr et al. 2004, 2006). Specificity determines the number of symbiotic combinations which the fungus is able to form with the photobionts available in a habitat. Local conditions influence the fitness and abundance of a photobiont, i. e. its availability for an incoming fungus. Consequently, fitness of the whole lichen (the holobiont) is also affected by the local environment – a symbiotic combination flourishing in one habitat does not have to be

succesful in a different one. The switching of photobionts has been observed in many lichen-forming fungi, even in those exhibiting mainly vegetative reproduction, that theoretically did not have any reason to find a new photobiont because they carried their current alga in joint propagules (vertical transmission; Nelsen & Gargas 2008, Wornik & Grube 2010).

However, selection of the photobionts can be fully performed only under the conditions supporting high diversity of photobionts. In extreme habitats, where only several tolerant species are able to survive (low temperatures, high toxicity, etc.), the fungus must either be adapted to the local photobionts or it has to be less selective. Less selective, flexible mycobionts have a clear advantage in such environments. In Antarctica, the low selectivity was observed in cyanolichens (Wirtz et al. 2003) as well as green algal lichens (Romeike et al. 2002).

Moreover, the obvious decrease of specificity in harsh Antarctic conditions is represented by the case of *Lepraria borealis* (Engelen et al. 2010). Temperate European and North American populations of *L. borealis* associate typically with *Asterochloris* algae (my results, see Paper 3 in this thesis), however, in extreme Antarctic climate *L. borealis* cooperates, besides *Asterochloris,* with *Trebouxia* as well[5].

Multiple photobionts in a single thallus

Many cases of multiple photobionts in a single thallus have been described (Helms et al. 2001, Romeike et al. 2002, Guzow-Krzemińska 2006, Piercey-Normore 2006, etc.). A mixture of algal genotypes actually results from low selectivity of the mycobiont (at one time, in one place and one thallus, the fungus associates with different algae as primary photobionts). It may be an adaptive advantage to fluctuating environmental conditions (Engelen et al. 2010, Romeike et al. 2002), because it can serve as an effective resource of various photobionts which can be used in different climatic conditions. The fungus may also utilize several algal genotypes to achieve maximum photosynthesis under varying light levels, e.g. in the forest canopy (Piercey-Normore 2006).

[5] This finding represents a second record of *Trebouxia* from the genus *Lepraria*. Formerly, Takeshita (2001) identified *Trebouxia corticola* and *T. higginsiae* in specimens of *Lepraria* sp. However, these records were based only on morphological investigation and should be revised.

The specificity of the photobiont

The ability of one distinct photobiont to associate with various lichen-forming fungi is very common. Algal as well as cyanobacterial species or strains are often shared within a distinct lichen community (e.g. Beck 1999, Doering & Piercey-Normore 2009, Engelen et al. 2010, Rikkinen et al. 2002) but also among various lichens from different habitats and regions (e.g. Hauck et al. 2007, Piercey-Normore 2009, Piercey-Normore & DePriest 2001).

4 The object and the aims of the study

My study is focused on green algal photobionts – lichenized algae from the genus *Asterochloris* (as photobionts of *Lepraria* and related fungal taxa). In a lesser extent I also studied photobionts belonging to the genus *Trebouxia*.

The overall aim of the thesis was to explore the identity of photobionts in selected lichen taxa or community in order to obtain data on their diversity, specificity and ecology.

The principal objectives can be characterized as follows:

1. To investigate the chloroplast morphology in lichenized algae *Asterochloris* and *Trebouxia*, in the context of their species identification; and to describe differences of chloroplast structure in lichenized and cultured (free-living) photobionts (*Paper 1*)**.**

2. To inventorize the photobionts

a) in lichen community growing under specific environmental conditions – metal-polluted habitats (*Paper 2*)**,**

b) in selected lichen taxa – genera *Lepraria* and *Stereocaulon* (*Paper 3*)**.**

The purpose of the inventories was to reveal the identity and diversity of the photobionts, the specifity of the photobionts as well as the mycobionts, and the contribution of the photobionts to ecological requirements of lichens.

5 Outline of the thesis

All the objectives as well as outputs of the thesis were linked by their object – *Asterochloris* algae, and by the basal goal – identification of the photobionts as an essential technique for exploration of their diversity.

During the first period of the study, I searched for the possibilities for identification of the *Asterochloris* algae. The culturing and morphological investigation of photobionts were combined with the molecular methods (*Papers 1*, *4*). The culturing experiments confirmed the possibility of obtaining axenic strains of photobionts from various lichens. Examination by confocal microscopy revealed a structural variability of chloroplast in different life stages of photobionts and the difficulty of morphological identification of *Asterochloris* algae. Sequencing of DNA appeared to be an easily applicable and more accurate method, and therefore it was used for the consequent research of algal diversity (*Papers 2*, *3*, *5*). These explorations of photobiont diversity in lichen-forming fungi representing several genera (especially *Cladonia*, *Lepraria* and *Stereocaulon*) yielded many interesting results about the ecology, specificity and other aspects of *Asterochloris* algae including several new findings (see below). All studies provided the information about the functioning of the partnership between lichen-forming fungi and their autotrophic partners.

My thesis is composed of three principal and two additional papers (see List of Papers and the note below[6]).

In this outline, I try

I) to briefly present the main ideas and results of the papers (objectives),

II) to discuss the results from the perspective of the essential topics of photobiont research (as outlined in Introduction).

[6] Note: The *papers 4* and *5* contain rather taxonomical study of *Asterochloris* algae. They were included in Ph.D. thesis of Pavel Škaloud. However, they arose as a part of the study of *Lepraria* and *Cladonia* photobionts, I contributed to them as a second author (my contribution is specified in chapter 8 Author´s contribution). So, I included them to my thesis as additional papers.

5.1 The main ideas and results of three principal papers

Morphology of the chloroplast in Asterochloris and Trebouxia (Paper 1)

A microscopical study of green-algal photobionts representing genera *Asterochloris* and *Trebouxia* was performed to observe the changes in chloroplast structure in lichenized algae.

Using confocal microscopy, remarkable variations were detected in different ontogenetic, physiological and ecological stages of a photobiont. During the study of various developmental states in four algal strains representing two species (*Asterochloris* sp. and *Trebouxia incrustata*), significant differences in the chloroplast structure of lichenized and cultured (free-living) algae were detected. The chloroplast of lichenized alga within lichen thalli was found to be more compact and rather simple in comparison with cultured alga, the pyrenoid/chloroplast ratio was approximately double in the lichenized form.

Moreover, algae were observed periodically during the process of their liberation (isolation) from the lichen thallus (they were isolated using „fragment method"). They exhibited gradual changes in chloroplast shape and structure during successive phases of cultivation – from the relatively simple lichenized form to the complex lobate chloroplast with several ontogenetic (morphological) stages observable in mature axenic cultures.

The morphological determination of photobionts was found to be rather difficult and unreliable (although interspecific differences in the chloroplast of some *Asterochloris* species were found – *Paper 4, 5*).

Photobionts in lichens from metal-rich substrates (Paper 2)

The photobionts of terricolous lichen community from metal-polluted habitats (mainly copper mine spoil heaps) were analysed to obtain the information about the diversity of lichenized algae, as well as the specificity and selectivity in lichen associations.

Only a few adapted algae were supposed to be abundant in fruticose lichens from these toxic locations. However, several *Asterochloris* lineages and one *Trebouxia* species were detected using phylogenetic analysis of algal ITS sequences. All these lineages represented photobionts with broad ecological amplitude and worldwide distribution.

This surprising diversity of photobionts could be explained by either subcritical concentrations of heavy metals absorbed by fruticose thalli that is not high enough to considerably affect photobionts, or by high toxitolerance of all detected photobiont lineages.

In several taxa of pioneer lichens, low specificity, as well as selectivity of lichen-forming fungi was revealed. They associated with several algal lineages (even within one thallus). We suggested that due to their intensive dispersal through vegetative propagules, such pioneer lichens can serve as an important photobiont transferring system.

Environmental preferences in lichenized algae – the study of photobionts in the genus Lepraria and Stereocaulon (Paper 3)

The molecular study of photobionts from selected taxa of Stereocaulaceae (*Lepraria* and *Stereocaulon*) was performed to investigate their diversity and ecological preferences.

Using phylogenetic analysis based on the concatenated set of ITS rDNA and actin type I locus sequences, 13 well resolved clades of *Asterochloris* were found to be associated with 16 *Lepraria* and 8 *Stereocaulon* phenotypic species.

Each photobiont lineage was associated with at least two and up to eight *Lepraria* or *Stereocaulon* taxa. Consequently, the majority of *Lepraria* species were associated with more than one photobiont genotype suggesting rather low specificity and intensive switching of photobionts in these asexual fungi.

Photobionts from particular algal clades were found in taxonomically different but ecologically similar lichens. The rain and sun exposure was the most significant environmental factor, clearly distinguishing the *Asterochloris* lineages. Photobionts from ombrophobic and ombrophilic lichens were clustered in completely distinct clades. Moreover, two photobiont taxa were obviously differentiated based on their substrate and climatic preferences. These environmental preferences of photobionts control the ecology of lichens and lead to the existence of specific lichen guilds.

5.2 The results in the perspective of the main topics of photobiont research

The identity of photobionts in lichens studied

The identification of photobionts represented the basal goal of all studies performed. The employment of DNA sequencing and consequent phylogenetic analysis (ITS rDNA and actin type I locus) turned out to be necessary for classification of the photobionts within lichen thalli as well as for specification of the identity of cultured algal strains.

Photobionts from 154 lichen specimens representing 39 fungal species within 7 genera were succesfully investigated using microscopy and/or molecular technics: *Lepraria* – 104 samples, *Cladonia* – 35, *Cetraria* – 4, *Diploschistes* – 4, *Lecidea* – 1, *Stereocaulon* – 3, *Xanthoparmelia* – 1 (Appendix 2 – List of specimens investigated)[7]. A number of investigated lichen taxa have not been analysed for their photobionts so far (*Cladonia foliacea*, *C. rei*, *Lepraria alpina*, *L. crassissima*, *L. granulata*, *L. membranacea*, *L. neglecta* and *L. rigidula* – see Appendix 1).

Members of the fungal genera *Cladonia*, *Diploschistes*, *Lepraria* and *Stereocaulon* were found to be associated with algae from the genus *Asterochloris*; lichens from the genera *Cetraria*, *Lecidea* and *Xanthoparmelia* contained *Trebouxia* in their thalli. These observations agreed with previous premises of lichen specificity at the level of genera (Rambold et al. 1998). However, I could not confirm the findings of Friedl (1987) who identified both *Asterochloris* and *Trebouxia* algae in parasitic lichen *Diploschistes muscorum*. Four examined samples of well developed, mature thalli of *Diploschistes*, contained only the *Asterochloris* algae originating probably from the host *Cladonia* species.

The diversity of photobionts

From a total of 154 lichen specimens obtained (see Appendix 2), only 19% of photobiont sequences clustered with current *Asterochloris* or *Trebouxia* morphospecies (*A. glomerata* – 14 samples, *A. irregularis* – 3, *A. phycobiontica* – 9, *T. incrustata* – 2, *T. decolorans* – 1). The remaining 81% of sequences belonged to undescribed species (four of them were affiliated with provisional species *T. hypogymniae* Hauck & Friedl ined.).

[7] Additionally, photobionts of *Hertelidea botryosa* (1 sample) and *Xanthoria parietina* (1 sample) were examined beyond the published studies. Photobiont of *H. botryosa* belonged to *Asterochloris glomerata*, the sample of *X. parietina* contained *Trebouxia decolorans*.

Above all, the investigation of photobionts in the genus *Lepraria* brought new findings on species diversity of the genus *Asterochloris*. There are apparently many undescribed and undetected species. In samples from Europe and North America, photobionts belonging to 13 well resolved lineages were detected. Only one of them could be assigned to formally described species (*A. phycobiontica*), six of them have never been reported (i.e. no previously reported sequences fell into these clades – A3, A4, A5, A9, A10, A11 sensu *Paper 3*). Moreover, there were several additional samples with unclear phylogenetic position which pottentially represented other phylogenetic lineages (OP 518, OP 544, OP 866, OP 786; see Fig. 1 in *Paper 3*).

All new lineages were revealed during the investigation of lichen taxa poorly examined for their photobionts (*Lepraria* species from different habitats). Thus, although the number of photobionts will be probably lower then that of mycobionts (six new *Asterochloris* lineages were found in nine fungal morphospecies, at least until now), we can expect many new discoveries of lichenized algae and cyanobacteria from unexplored lichen taxa or/and biotopes.

Biogeography

Although I collected lichen samples especially in Central Europe, some additional collections from other regions together with data from GenBank and published studies, allow to estimate the distribution tendencies of some photobiont lineages.

Seven from a total of 17 phylogenetically well resolved clades (15 *Asterochloris* and 2 *Trebouxia*) have been found in two or more continents. Four lineages were detected in lichen samples from Europe and North America (*T. hypogymniae* ined., *T. incrustata*, *Asterochloris irregularis*, clade A13 sensu *Paper 3*), one from Europe and Central America (clade A6) and two from Europe, North America and Asia (*A. glomerata*, clade A12).

These data are certainly influenced by undersampling of Asian, African as well as South American continent, nevertheless, they at least suggest an intensive exchange of photobionts among the European and North American lichens.

It is not obvious, whether this exchange is recent or late. The opinions about intercontinental dispersal of lichens are contradictory (cf. Prinzten 2008). However, the Europe and North America share a high number of lichen taxa associated with forenamed photobiont lineages (for example, most of *Cladonia* and *Stereocaulon* species associated with

A. glomerata grow in both continents). Similarly to photobionts, a number of European and American lichen-forming fungi have been found genetically identical or very close, supporting the hypothesis of recent gene flow (e.g. Beiggi & Piercey-Normore 2007, Blanco et al. 2004, Buschbom 2007, Höchberg et al. 2002, Nelsen & Gargas 2009, Thell 1999). Many of these lichens disperse intensively by vegetative propagules, especially soredia, which can provide an effective transfer of photobionts over large distances.

Ecology

The important question of the thesis was how much photobionts influence the ecology and distribution of lichens due to their own environmental preferences. Theoretically, if mycobionts had the ability to create stable conditions inside the thalli, then all photobionts would occur in all compatible lichen-forming fungi regardless of the habitat type.

However, the obtained data indicate that photobionts were distributed according to certain patterns – some of them occured only in specific habitats and lichens growing there.

During the investigation of *Lepraria* lichens, the distribution of some photobiont lineages was determined by water conditions (rain exposed/rain sheltered surfaces), climatic conditions (mountains/lowlands) or type of the bedrock (e.g. granite/shale). Such photobionts maintained remarkably their ecological requirements, so that they occured only in specific stands although the associated mycobionts can have a broader ecology, e.g. psychrophilous *Asterochloris* clade A1 associated, except the mountain species, with *Lepraria caesioalba* and *L. borealis* which can grow also in lowlands. The existence of specific lichen guilds is based only on the photobionts that are not able to cross a border outlined by certain environmental factor (temperature, water conditions, etc.).

On the other hand, a study of the terricolous lichen community from heavy-metal-polluted habitats revealed photobionts with very broad ecological amplitude (*Asterochloris glomerata*, *A.* clade C and D sensu *Paper 2*, *Trebouxia hypogymniae* ined.). Besides the tolerance to toxic heavy metals, these algae have been detected in lichens growing on substrates with different pH as well as in various climatic conditions.

The existence of such euryecious photobionts seems to be very important for the lichens survival. The fungus housing such algae can survive under changing environmental conditions. Moreover, vegetative diaspores containing tolerant photobionts have a greater

probability to survive and prosper at an impact point. Unsurprisingly, these photobionts were detected in a number of pioneer lichens responsible for fast colonization of new habitats.

Specificity and selectivity

Mycobionts

From a total of 20 fungal taxa (phenotypic species) represented by two or more investigated specimens, 14 taxa were associated with more than one photobiont lineage. Moreover, in four *Cladonia* species, individual lichen thalli contained multiple photobiont genotypes. Although all fungi were strictly specific either to *Asterochloris* or to *Trebouxia*, at the level of species, their specificity was rather low (or it was not high). Thus, this study confirmed the results of previous studies that there probably exist only a few lichens with really high specificity.

Selectivity in the sense of „the frequency of associations among compatible partners“ [8] could only be estimated in some lichens from heavy metal-polluted substrates that were represented by several samples from the same habitat or locality. In all cases, the selectivity of the mycobionts corresponded to their specificity. For example, in four samples of *Cladonia subulata* from one locality of sedimentation basin in Chvaletice, four *Asterochloris* lineages were detected. Eight samples of *Cladonia rei* from metal polluted localities contained four different photobionts with similar frequencies. These cases illustrate the low specificity of these *Cladonia* species and simultaneously their very low local selectivity (nevertheless, in case the future studies reveal that *C. subulata* and *C. rei* associate worldwide with 30 algal lineages, then we will say they were rather highly selective in our localities).

Photobionts

The observed specificity of the photobionts fulfilled the preliminary expectations. Not a single algal lineage associated with just a single fungal species. The most specific algal lineage was a clade shared by two closely related species (*A.* clade A8, *Paper 3*). The least specific lineages associated with 6 or more fungal species (clades C and D sensu *Paper 2*, clades A7 and A12 sensu *Paper 3*).

The data show that photobionts are frequently shared among closely related fungi (within one genus or intrageneric group), but also among unrelated fungi within a single local

[8] in *Paper 2*, the term selectivity was used more generally (in sense of Beck et al. 2002, p. 323)

community or even in geographically distant lichens. Moreover, the investigation of *Lepraria* lichens confirmed the existence of intensive photobiont sharing among completely sterile lichens.

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7. List of papers

My thesis is based on the following five papers, mentioned in the previous text as *Papers 1–5*.

Principal papers:

Paper 1

Peksa O. & Škaloud P. (2008): Changes in chloroplast structure in lichenized algae. – *Symbiosis* 46: 153–160.

Paper 2

Bačkor M., **Peksa O.**, Škaloud P. & Bačkorová M. (2010): Photobiont diversity in lichens from metal-rich substrata based on ITS rDNA sequences. – *Ecotoxicology and Environmental Safety* 73(4): 603–612.
DOI: 10.1016/j.ecoenv.2009.11.002

Paper 3

Peksa O. & Škaloud P.: Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). – ~~*resubmitted*~~ *in Molecular Ecology*.

Additional papers:

Paper 4

Škaloud P. & **Peksa O.** (2008): Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta). – *Biologia* 63/6: 873–880.
DOI: 10.2478/s11756-008-0115-y

Paper 5

Škaloud P. & **Peksa O.** (2010): Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta). – *Molecular Phylogenetics and Evolution* 54/1: 36–46.
DOI: 10.1016/j.ympev.2009.09.035

8. Author´s contribution

***Paper 1**:* **O. Peksa** was responsible for the idea and planning the study. He collected and determinated the lichen samples, isolated and cultivated algae, performed the light and confocal microscopy, DNA isolation and following PCR reactions, he wrote the paper with small contribution of P. Škaloud, who helped with confocal microscopy, sequences processing and improving the manuscript text as well.

***Paper 2**:* M. Bačkor was responsible for the idea. The study was planned jointly. **O. Peksa** collected and determinated the lichen samples from Chvaletice, determinated all lichen samples, performed DNA isolation and following PCR reactions in part of samples, prepared and wrote: part of chapter „ *2. Material and methods*“, part of „*3. Results*“, whole „*4. 1. Photobiont identity*“, part of „*4. 2. Photobionts and heavy metal pollution*“, whole *4. 3. Fungal selectivity and multiple algal genotypes*“, whole *4. 4. Photobionts in lichen communities*“, and helped to improve the remaining manuscript text. P. Škaloud performed the molecular analyses. M. Bačkorová helped with DNA isolation and following PCR reactions. All co-authors helped to improve the text of the manuscript.

***Paper 3**:* The study was planned jointly. **O. Peksa** collected and determinated the lichen samples, performed DNA isolation and following PCR reactions in the majority of samples, prepared and wrote most of the manuscript. P. Škaloud performed the molecular analyses and tests of the phylogenetic signal and the contingency of character evolution in R and BayesTraits, he wrote the appropriate parts of Methods and Results.

***Paper 4**:* P. Škaloud supervised this study (planning, performing confocal microscopy and molecular analyses, writing the paper – see his Ph.D. thesis). **O. Peksa** collected and determinated the lichen samples, isolated and cultivated the algal strains and helped with DNA isolation and following PCR reactions, helped to improve the text of the manuscript.

***Paper 5**:* P. Škaloud supervised this study (planning, performing confocal microscopy and molecular analyses, writing the paper – see his Ph.D. thesis). **O. Peksa** collected and determinated the lichen samples, isolated and cultivated the algal strains, performed DNA isolation and following PCR reactions, prepared and wrote the chapter „*4. 3. Specificity of Asterochloris to lichen-forming fungi*“ and „*Supplementary Table 1*“, helped to improve the text of the manuscript.

Paper 1

Peksa O. & Škaloud P. (2008): Changes in chloroplast structure in lichenized algae. – *Symbiosis* 46: 153–160.

Changes in chloroplast structure in lichenized algae

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Abstract

Chloroplast morphology represents a basic characteristic in the systematic classification of trebouxioid algae. However, in different ontogenetic, physiological and ecological stages chloroplasts may vary markedly. Various developmental states of two algal species (*Asterochloris* sp. and *Trebouxia incrustata*) isolated from four lichens (*Cladonia foliacea*, *Lecidea fuscoatra*, *Lepraria* sp., *Xanthoparmelia conspersa*) were examined by confocal microscopy for variations in chloroplast structure. Distinct differences were detected between the chloroplast structure of cultured and lichenized photobionts. Remarkable chloroplast changes were observed during the process of algal liberation from the lichen thallus.

Keywords: Confocal laser scanning microscopy, green algae, isolation, Lecanorales, mycobiont, pyrenoid, symbiosis, thallus fragment method

1. Introduction

Lichens are symbiotic organisms composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, that may be either a green alga or cyanobacterium (Nash III, 1993). In symbiotic organisms, the partners are typically adapted to coexistence to varying degrees. The success of algae and fungi as lichen symbionts implies a set of specialized and possibly irreversible characteristics (Ahmadjian, 1992). Both symbionts undergo a variety of structural, physiological, and biochemical modifications as a result of lichenization (Galun, 1988). Cyanobacterial as well as green algal photobionts are changed by the influence of their fungal partner. Due to these changes, identification of photobionts directly in the lichen thallus is often impossible (Friedl and Büdel, 1996). However, the changes of the algal cells are not permanent and when the cells are freed from the fungal hyphae they revert to their original size and characteristics after several divisions (Ahmadjian, 1992).

Morphological differences between symbiotic and cultured photobionts have been reported in many studies (see references in Bubrick, 1988; further e.g. Ahmadjian, 1992; Davis and Rands, 1993; Tschermak-Woess, 1995a,b). Commonly, a remarkable reduction of cell size is found in a majority of eukaryotic bionts. Filamentous algae and cyanobacteria are generally reduced to unicellular forms. Both sexual and asexual reproductive strategies of algae and the cell division of cyanobacteria are usually modified. At the subcellular level, modifications of size, structure, as well as the number and distribution of some organelles and cellular structures were detected (e.g. chloroplast, thylakoid, pyrenoid, pyrenoglobuli, dictyosomes, etc.). Changes in cell wall chemistry and structure, and in the production of a gelatinous sheath have been observed in several cases. Cell envelopes and internal structures may be also directly affected by penetration of haustoria.

The degree of structural modification is dependent upon the type of photobiont and on the specific relationship between two particular partners (closeness of mycobiont-photobiont contact). However, these modifications may also be evoked by environmental conditions and physiological status of the biont.

This paper presents a microscopical study of photobionts from four different lichen taxa. The aims were twofold: 1) to demonstrate differences in chloroplast structure between the lichenized and cultured form of symbiotic algae from the genera *Asterochloris* and *Trebouxia* and 2) to document changes in chloroplasts during the unprompted liberation of algae from lichen thalli.

2. Materials and Methods

2.1. Material

The algal symbionts used in this study were isolated from four lichens with different types of thalli and systematic position: *Cladonia foliacea* (thallus heteromerous dimorphic; Cladoniaceae), *Lecidea fuscoatra* (heteromerous crustose; Lecideaceae), *Lepraria* sp. (homoiomerous leprose; Stereocaulaceae; in the collected specimen two species were detected: *Lepraria nylanderiana* and admixed *Lepraria* sp.), *Xanthoparmelia conspersa* (heteromerous foliose; Parmeliaceae).

All lichens were collected from the single rocky steppe slope near Máslovice in Central Bohemia, Czech Republic (alt. 280 m, 7. 1. 2006).

2.2. Isolation and cultivation of the photobionts

Photobionts were isolated by the thallus fragment method (Ahmadjian, 1993) as follows: small fragments of lichen thalli (cross-sections of heteromerous thalli, soredia from *Lepraria*) were plated onto agar slants in Petri dishes (BBM 3N - Bold's basal mineral medium according to Deason and old (1960) with three times the original amount of nitrogen) and incubated at 18°C, under an illumination of 20–30 mmol m^{-2} s^{-1} and a 16:8 h light-dark cycle. If fungal contamination occurred during the cultivation, the contaminants were carefully removed or the thalli fragments were transferred to new plates. After 2–3 weeks, groups of dividing algal cells were observed associated with some of the fragments. To obtain unialgal cultures, small populations of photobionts were transferred to BBM 3N and *Trebouxia* medium (according to Ahmadjian 1993). The isolates were cultivated under the conditions mentioned above. Cultured strains are maintained in the culture collection of O. Peksa at the Department of Botany, Charles University in Prague (strain numbers are included in Table 2).

2.3. Microscopy

The pure algal samples and fragments of lichen thalli were examined under light microscopy with an Olympus BX 51 with differential interference contrast optics, and by laser scanning confocal microscopy with a Leica TCS SP2 equipped with an Argon-Krypton laser using a 488 nm excitation line and AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion or 63x/1.2 water immersion objective fitted on the Leica DM IRE2 inverted microscope was used. A series of optical sections of chloroplasts were captured and used for 3D reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for visualization of the chloroplast structure. For the final processing of the confocal images and visualization of various chloroplast structures, Leica Confocal Software, version 2.61 (Leica Microsystems HeidelbergGmbH) and the Image J 1.37v and 1.38t program (Abramoff et al., 2004) were used. Images were finally resampled for print by IrfanView 3.98 (Irfan Skiljan, Vienna University of Technology).

2.4. Observation scheme

Photobionts were observed A) within desiccated fragments of the lichen thalli; B) immediately after hydration of the fragments; C) within hydrated thalli (fresh thalli incubated

3 days in the same conditions as the isolates and cultures); D) during the process of their liberation from lichen thalli (at 3, 7, 11, 20, 30 and 40 days after plating thallus fragments onto agar).

2.5. DNA extraction, PCR, sequencing, BLAST search

Total genomic DNA was extracted from lyophilized cultures following standard CTAB protocols (Doyle and Doyle, 1987) with minor modifications. DNA was resuspended in sterile dH2O and amplified by the polymerase chain reaction (PCR). Amplification of the ITS of nuclear rDNA was from an algal-specific primer nr-SSU-1780-5' (Piercey-Normore and DePriest, 2001) and a universal primer ITS4-3' (White et al., 1990). For the amplification of the actin type 1 locus, we used the combination of algal specific primers a-nuact1-0645-5' and a-nu-act1-0818-3' (Nelsen and Gargas, 2006). PCR amplifications were performed using Red Taq DNA Polymerase (Sigma), cycling conditions for the ITS rDNA follows Piercey-Normore (2006), for Actin 1 locus Nelsen and Gargas (2006). PCR products were quantified on 1% agarose gel and cleaned with Genomed Jetquick Kit. Sequencing PCR was performed using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems), sequencing on an ABI 3100 Avant genetic analyzer sequencer in 50 cm long capillary. Homology between obtained sequences and those currently available in the NCBI database was assessed using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, NIH, http://www.ncbi.nlm.nih. gov/BLAST; Altschul et al., 1997) score system.

3. Results and Discussion

3.1. Identity of photobionts

For our investigation of photobionts, we chose four morphologically and taxonomical varied lichen taxa collected at the same stand. Interestingly, we distinguished only two species of symbiotic algae within the chosen lichens. Based on morphological and biological characteristics (Table 1) as well as ITS rRNA and partial Actin 1 sequences, we identified *Asterochloris* sp. in thalli of *Cladonia foliacea* and *Lepraria* sp., and *Trebouxia* incrustata Ahmadjian ex Gärtner in *Lecidea fuscoatra* and *Xanthoparmelia conspersa* (photobionts of lichen taxa *C. foliacea*, *L. fuscoatra* and *X. conspersa* are being described here for the first time). Both pairs of photobiont strains were genetically highly similar. Sequence similarities of *Asterochloris* strains were 100.0% in ITS rDNA (528 total nucleotides / 0 different

nucleotide positions) and 99.8% in the Actin type 1 locus (517/1). Similarly, ITS rDNA similarity between *Trebouxia* photobionts was 99.6% (707/3). The findings of only two photobiont strains in four diverse lichens with different predominant reproduction mode at one stand supports a theory of photobiont-sharing among several mycobionts at one locality as described by Beck et al. (2002).

We confirmed that identification of photobionts from the genera *Asterochloris* and *Trebouxia* on the basis of morphological characters is difficult, and molecular methods are advisable for accurate determination. Based on a combination of microscopic features, strains isolated from *C. foliacea* and *Lepraria* sp. resemble species belonging to *Asterochloris* s. str. in many respects. Of particular note was the parietal position of chloroplasts prior to cell division, observed in both investigated strains, that constitutes one of the main discriminative features of the genus (Ahmadjian, 1960; Hildreth and Ahmadjian, 1981; Friedl and Gärtner, 1988; Škaloud and Peksa, 2008). The spherical shape of the cells, chloroplast with incisions running closely to the naked pyrenoid and the great number of aplanospores (most often 64 in sporangium) closely fit the description of *Trebouxia excentrica* according to Gärtner (1985). However, a BLAST search for the ITS rRNA sequences of both strains listed only ≥97% homology with the authentic culture of *T. excentrica* UTEX 1714 (AF345433; Piercey-Normore and DePriest, 2001). By contrast, the same BLAST search listed one sequence sharing ≥99% homology with the query. This sequence was obtained from an unidentified strain of *Asterochloris* sp., isolated from lichen *Stereocaulon saxatile* (DQ229886; Nelsen and Gargas, 2006).

The strains from *L. fuscoatra* and *X. conspersa* have characteristics similar to *T. incrustata* and/or *T. jamesii*. They are distinguished by deeply lobate chloroplasts with fine lobes (crenulate) to larger oblong ridged lobes (resemble rips by *T. jamesii*) and naked pyrenoid. We also observed nonsynchronous divisions in autosporangia, as mentioned by Gärtner (1985) as a typical feature of *T. incrustata*. A BLAST search for the ITS rRNA sequences of both photobionts from *L. fuscoatra* and *X. conspersa* listed 18 sequences sharing ≥99% homology with the query. Among many sequences derived from uncultured and unidentified photobionts, a sequence of the authentic strain of *T. incrustata* UTEX 784 was displayed (AJ293795; Helms et al., 2001).

3.2. Chloroplast changes

Microscopic investigation of photobionts from both lichenized and cultured stages was carried out for determination of chloroplast morphology in different ecological and physiological conditions as well as ontogenetic stages. We observed distinct differences among various life stages of algal cells and confirmed an influence of different living conditions on chloroplast structure. Chloroplast morphology was predominantly dependent on physiological (dryness/dampness) and ecological status (lichenized vs. free-living) in both investigated algal species. Ontogenetic changes were obvious, especially in cultured (free-living) forms of photobionts.

In the lichenized algae, a chloroplast is usually modified in some way. Within a desiccated lichen thallus (herbarium specimen) it is highly deformed, and distinctly compressed within a dehydrated protoplast; the pyrenoid is small, and poorly visible in only a portion of cells (Fig. 1a).

A similar effect was observed by De los Ríos et al. (1999) in desiccated thalli of *Lasallia hispanica* and *Parmelia omphalodes*. They described very collapsed, star-like shaped cells with a small pyrenoid full of pyrenoglobuli. However, such deformations of the chloroplast is an obvious result of physiological drought. Immediately after hydration of a dry thallus chloroplasts are very rapidly changing into globular compact forms without lobes or with poorly visible lobes (Fig. 1b). In this form, the pyrenoid is clearly observable including penetrating thylakoids. However, this rehydration is possible only in relatively fresh specimens. As demonstrated by Honegger (2003), protoplast of the algal cells in thalli stored several years at room temperature failed to rehydrate and appeared irregular in shape (chlorophyll's autoflorescence is also lost).

According to our experience with isolation of photobionts, their viability varies in different types of lichen thalli: it is shorter in homoiomerous (leprose) thalli than in heteromerous. Viability (possibility of isolation and cultivation) of photobionts from thalli of *Lepraria* spp. stored at room temperature is likely up to 6 months at the most. By contrast, algae from lichens with heteromerous thalli are viable after storage for several years.

Physiologically active cells within a wet lichen thallus (after 3 days in wet conditions) have more developed chloroplast as compared with recently hydrated cells (Fig. 1c). The chloroplast occurs in several morphological stages with different degrees of lobation, but in comparison with cultured algae it is rather simple. Ahmadjian (1992) described changes of

algal cells during resynthesis of the lichen thallus. The cells of the Trebouxia photobiont from Acarospora fuscata became smaller after they were enclosed in fungal pseudoparenchyma, chloroplasts enlarged and filled more of the cell, and the pyrenoid became larger and clearer. Our observations confirmed this pattern. Lichenized algae have more compact, less structured chloroplasts and the pyrenoid/chloroplast ratio is approximately double that of free-living algae. The mechanism of conversion from an intricate structure to a simple form is unclear, but it is evidently evoked by the mycobiont. The fungus can influence the alga either physically (direct contact, haustoria) or physiologically (chemically). During our investigation, we did not find an obvious relationship between physical contact of bionts and deformation of chloroplasts. For example, we observed very different haustoria types in *L. fuscoatra* and *X. conspersa* (deeply penetrating intracellular haustoria and shallow invaginated intraparietal haustoria, respectively), however, photobionts in both lichens had very similar changes in their chloroplasts. Deformation of chloroplasts is more likely to be caused by a physiological influence of the mycobiont unrelated to mechanical impact. However, very little is known to date concerning the chemical (hormonal) activity between the bionts.

In all studied photobionts, we further observed remarkable changes in the internal chloroplast structure, namely a fluctuation in the amount of starch. Jacobs and Ahmadjian (1971) described changes in starch volume that were dependent upon water content in the photobiont of *Cladonia cristatella*. In wet conditions algae produce starch which is deposited in chloroplasts, and then in dry periods the starch disappears from algal cells. In our confocal images, starch is clearly observable as a dark area around the pyrenoid, therefore we were able to observe its fluctuation in different stages of algal cells. After approximately 10 days of cultivation, the cells contained the highest volume of starch (the majority of the chloroplast is filled by a starch area around the pyrenoid). By contrast, the chloroplast in dry and immediately hydrated cells seems to form a compact mass without starch (compare Figs. 1a,b with Fig. 2b).

During the liberation of photobiont cells from the lichen thallus, the chloroplast changed significantly in its shape and structure (Figs. 2, 3). Asterochloris strains in particular underwent distinct changes. The process of liberation, when a new generation of algal cells is fully developed and liberated from a fungal hyphae, took about five weeks. We didn't observed any differences in behavior of related strains from different lichens (timing as well

as morphological changes were almost identical). One of the remarkable chloroplast modifications takes place around the 10th day after the inoculation of a thallus fragments onto agar based medium (while still in the lichenized generation of algal cells). Chloroplasts of all cells in the population appear richly lobate and simultaneously contain a large volume of starch (Fig. 2b). During this initial period of being released from lichen thalli algae evidently adapt themselves to the new conditions of light and hydration (~ lag phase) and the decreasing influence of the mycobiont, which withers away due to unsuitable conditions (continuous moisture).

Approximately on the 20th day of cultivation, algal cells enter a phase of intensive division, characterized by the dominance of autospore (*Trebouxia*) or aplanospore (*Asterochloris*) packages (Figs. 2c, 3c). It is remarkable that during this phase algae maintain the mode of reproduction typical for their lichenized state: especially in Trebouxia we observed autosporangia with only 8 spores 20 days after the inoculation, however, aplanosporangia with 16–32 spores and zoosporangia with 64 spores did occur in mature cultures. The influence of the mycobiont evidently persists despite its considerable weakening under unsuitable conditions. Therefore, in parallel with chloroplast structure, the mode of reproduction noticeably changes during the formation of a new algal generation.

Mature cells of the new generation (30 and 40 days after the inoculation) were characterized by very different chloroplast shape compared with the lichenized generation. Moreover, several ontogenetic (morphological) stages of the chloroplast were observable in mature cultures (Figs. 2d, 3d). Each strain was characterized by either predominance or total absence of specific morphogenetic traits of the chloroplast (e.g. the photobiont of *C. foliacea* forms predominantly chloroplasts with unbranched lobes extended longitudinally at their ends – Fig. 2d; the strain from *L. fuscoatra* formed neither flattened nor extremely small fine lobes observed by strain from *X. conspersa* – Fig. 3d).

In addition to the investigation of morphological changes, it would be very interesting to observe physiological changes in photobionts. Green and Smith (1974) investigated physiological differences between lichen algae in symbiosis and following isolation. They found almost immediate changes in the physiology of algae arising after isolation from a lichen thallus (i.e. little 14C release, high incorporation into ethanol-insoluble materials, low incorporation into simple carbohydrates). In contrast to the fragment method of isolation, they liberated algal cells from fungal hyphae very quickly through successive centrifugations of 30

to 60 minutes. However, when compared with the data of Richardson and Smith (1968), their results showed some residual effect of the mycobiont on the isolated algae (in contaminated or less washed preparations). Thus, the mycobionts' influence on algae probably decreases gradually when the fragment isolation method is used. It would be advisable to investigate in detail the changes occurring in the chloroplast during the synthesis of a lichen thallus. When does the change from the lobate chloroplast of free-living algae to the simple lichenized form appear? It may already develop in the initial stage of contact between bionts (envelopment of the algal cells by fungal hyphae), but it seems more likely that it may arise during formation of the pseudoparenchymatic tissue in the first true lichenized unit.

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Table 1. Morphological and biological characteristics of investigated photobiont strains.

	***Asterochloris* sp.**		***Trebouxia incrustata***	
characteristic	**cultured**	**lichenized**	**cultured**	**lichenized**
vegetative cells	spherical (12.5–23 μm)	spherical or ellipsoidal (5–17.5 μm, 10–11 × 15–17.5 μm; in *Lepraria* only spherical!)	spherical (12.5–22.5 μm)	ellipsoidal or ovoid (12.5–15 × 17.5–20 μm) in *L. fuscoatra*, predominantly spherical in *X. conspersa* (6.5–17.5 μm)
cell wall	to 1 μm thick, wall thickening to 2.5 μm	–	to 1 μm thick, wall thickening to 2.5 μm	–
chloroplast	lobate: lobes simple at first, less or more incised and extended in terminal part; later thinner, curved and branched; prior to cell division (aplano/zoosporogenesis) in parietal position	compact, crenulate (short picked lobes) or with shallow incised flat lobes	lobate: crenulate (small rounded lobes) or with large oblong ridged lobes (incised to 1/4 of chloroplast average)	compact to lobate (simple, less or more oblong lobes)
pyrenoid	mainly single, naked, occurring in most compact part of chloroplast, surrounded with more or less distinct starch area	distinctly penetrated by one or more thylakoids	single or several, naked; in young cells and in a part of mature cells surrounded with distinct starch area	starch area often very large
nucleus	single, in parietal position (occurring in invagination of chloroplast)	–	single, in parietal position (occurring in invagination of chloroplast)	–
reproduction	aplanospores: 64 (128) in sporangium, zoospores (narrowly to broadly drop shaped, 5–10 × 2.5–4.5 μm, with two anterior flagella to 12 μm in length, apical stigma)	aplanospores: 4–16 in sp.	autospores: 4–16 in sp., often with non-synchronous division; aplanospores: 32 (64) in sp.; zoospores (narrowly drop shaped, 4–5 × 3 μm, with two anterior flagella about 7 μm in length, small apical stigma)	autospores: 4–8 in sp., non-synchronous division very frequent

Table 2. List of taxa used in this study, with collection information and GenBank accession numbers.

Algal species (Fungal Species)	Collection Number	Algal GenBank Accession Number	
		ITS	Actin
***Asterochloris* sp.** (*Cladonia foliacea*)	CLAD 1	**AM906016**	**AM906049**
***Asterochloris* sp.** (*Lepraria* sp.)	LEP 36	**AM900493**	**AM906046**
Trebouxia incrustata (*Lecidea fuscoatra*)	LEC 1	**AM920666**	-
Trebouxia incrustata (*Xanthoparmelia conspersa*)	PAR 1	**AM920667**	-

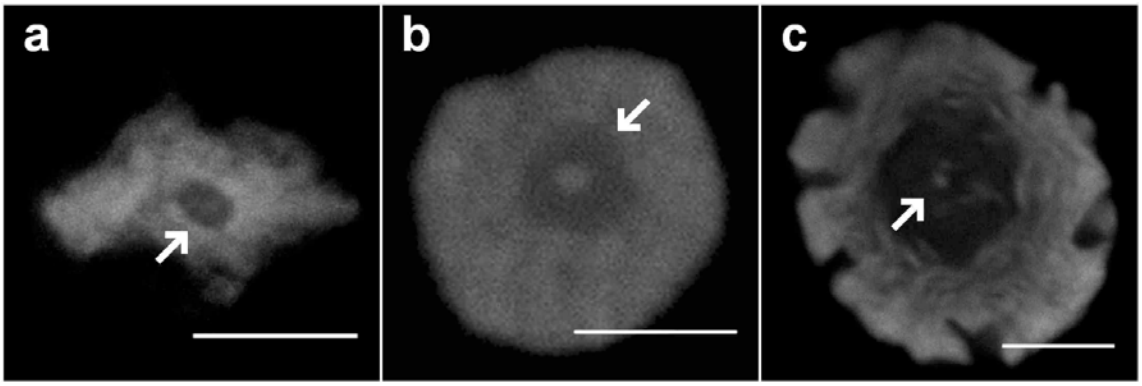

Fig 1. Effect of hydration to chloroplast structure of *Asterochloris* sp. from *Cladonia foliacea* – confocal sections of chloroplast. a) „star-like“ shaped chloroplast within a dessicated thallus (pyrenoid – arrow); b) globular compact chloroplast immediately after hydration – pyrenoid penetrated by thylakoid (arrow); c) lobate chloroplast within a wet (living) lichen thallus – pyrenoid distinctly penetrated by thylakoids (arrow) and surrounded with a starch sheath. Scale bar: 5 μm.

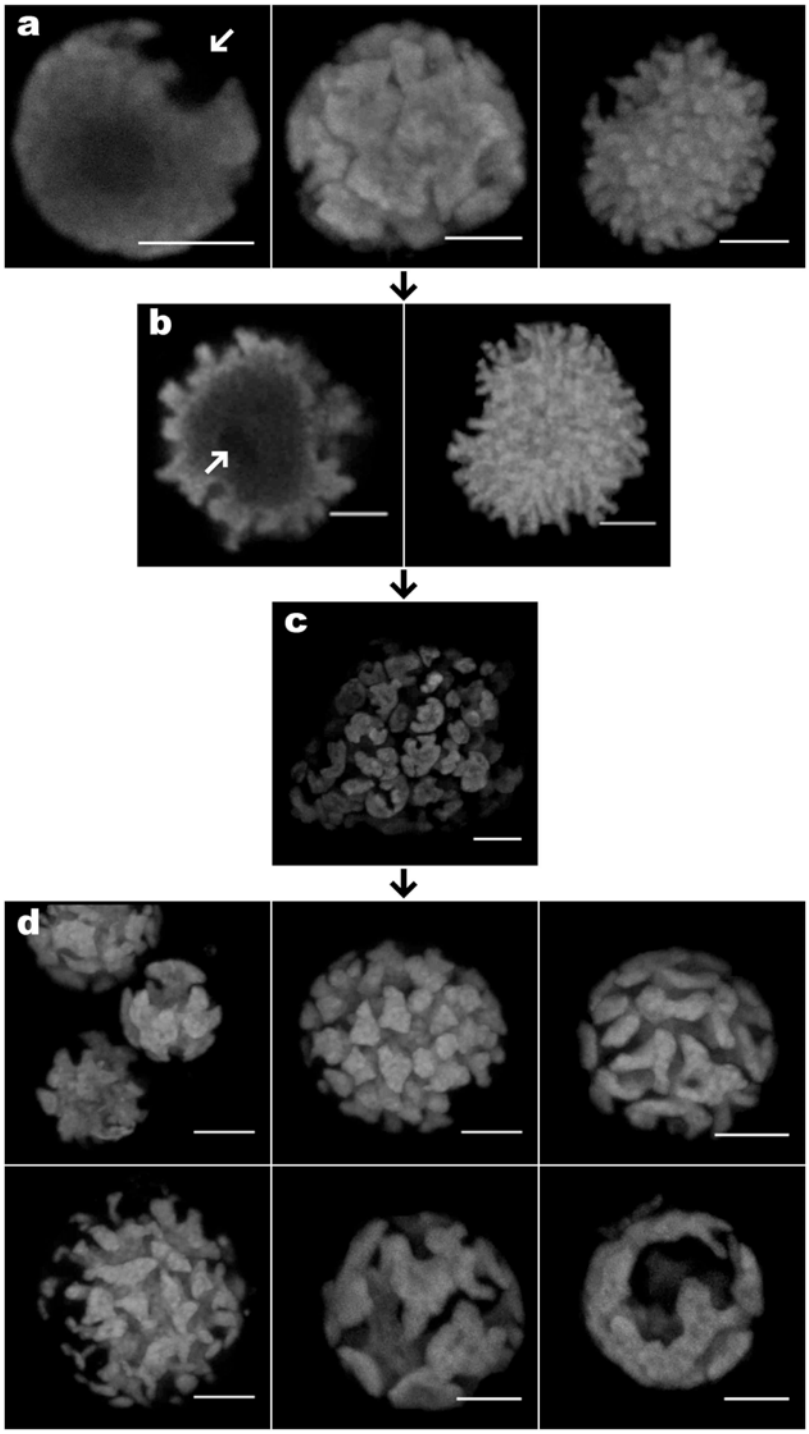

Fig 2. Chloroplast changes in *Asterochloris* sp. during the liberation from thalli of *Cladonia foliacea* (Clad) and *Lepraria* sp. (Lep) – confocal sections (first from the left in a, b) and maximum projections of chloroplast. a different chloroplast types of lichenized photobionts within a wet lichen thallus – pyrenoid and invagination for nucleus (arrow) well observable (Lep); b strongly lobate chloroplast on the 7th day of cultivation – the chloroplast is filled by an extensive dark stained starch area around the black pyrenoid (arrow) (Lep, Clad); c phase of intensive division (20th day) – mature aplanopores (Lep); d fully liberated generation of photobionts 30–40 days after the inoculation – several ontogenetic stages of chloroplasts with different morphologies occuring in culture (from the top left): simple lobate form of young cells; crenulate form; chloroplast with unbranched lobes extended longitudinally at their ends (elongate appearence in surface view); fragile T-shape lobes; deeply incised, broadly extended lobes branched in terminal part; chloroplast in parietal position prior to cell division (all figs. Lep). Scale bar: 5 μm.

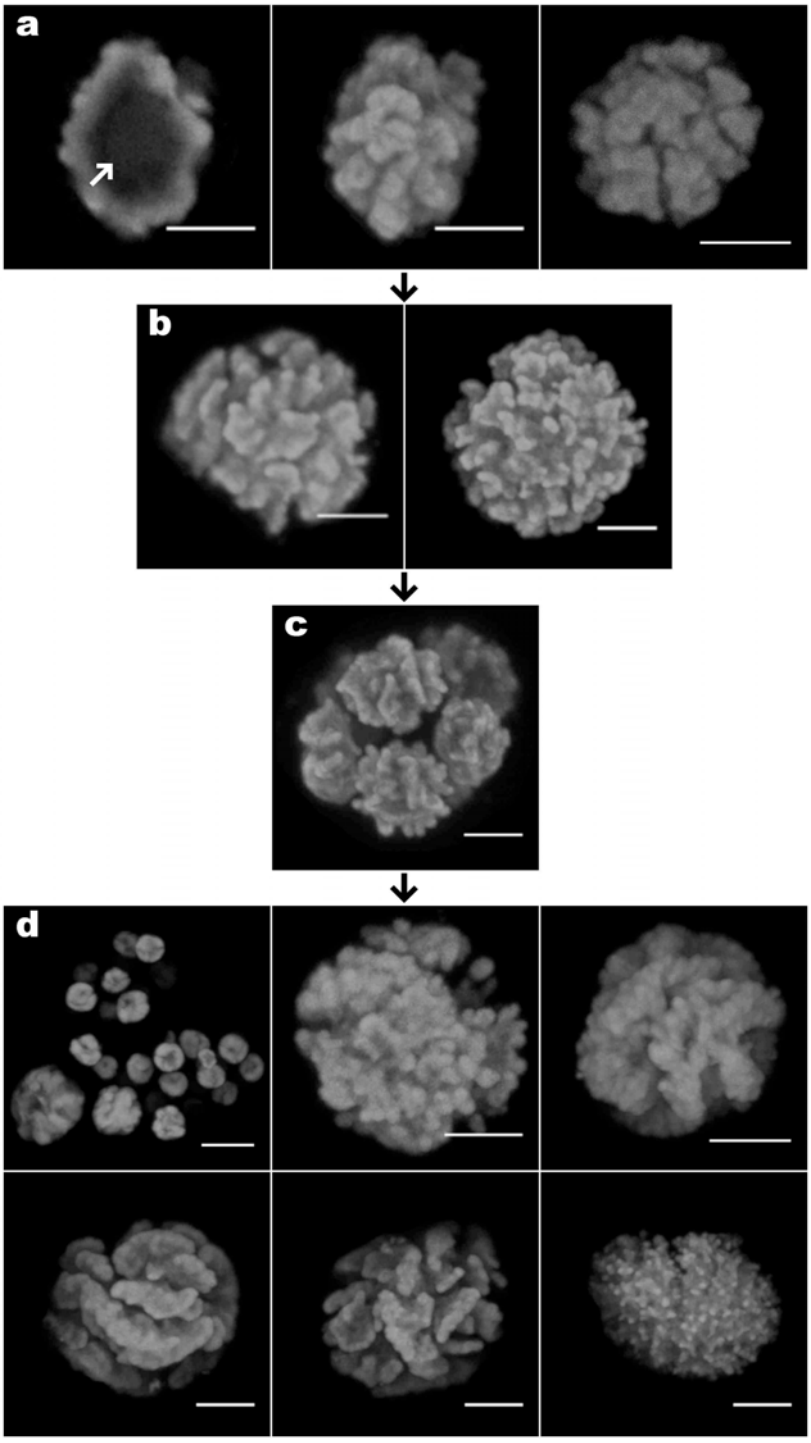

Fig. 3. Chloroplast changes in *Trebouxia incrustata* during the liberation from lichen thalli of *Lecidea fuscoatra* (Lec) and *Xanthoparmelia conspersa* (Xant) – confocal sections (first from the left in a) and maximum projections of chloroplast. a chloroplasts of lichenized photobionts within a wet lichen thallus – pyrenoid well observable (arrow) surrounded by a dark stained starch area (the first two Lec; Xant); b chloroplast 10 days after the inoculation (Xant); c phase of intensive division (20th day) – autospore package (Lec); d fully liberated generation of photobionts 30–40 days after inoculation – several ontogenetic stages of chloroplasts with different morphologies occuring in culture (from the top left): aplanospores and young cells (Lec); chloroplast with small rounded lobes (crenulate) (Lec); ridged lobes (2 types – Lec, Xant); flattened lobes (Xant); extremely small and fine lobes (Xant). Scale bar: 5 μm.

Paper 2

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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.

Keywords: algae, *Asterochloris*, Cu, heavy metals, metal toxicity, metal tolerance, *Trebouxia*

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 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 is toxic at high concentrations. Lichen as a symbiotic unit posseses 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 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 to 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 likely a 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 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 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* (Guschina 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 *T. 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 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 the comparison two localities: first non-polluted by heavy metals and situated near the Cu-mining area in central Slovakia, and the second, which is 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, Ľubietová-Podlipa, 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, 48º50´N, 20º56´E), Slovenské Stredohorie Mts. (Ľubietová-Podlipa, 570–700 m a.s.l., 48º45´N, 19º22´E) and Low Tatra Mts. (Špania dolina, 780 m a.s.l., 48º49´N, 19º08´E). The area around Ľubietová 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 these 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 (48º49´N, 19º03´E), Veľká Fatra Mts., central Slovakia, at approximately 500 m a.s.l.. This area belongs to Veľká Fatra National Park, and there is not 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 sulphuric 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 F_{254} 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 ^{o}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 λ_{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, 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 MgCl2, 1 µl of DNA (not quantified).

PCR and cycle-sequencing reactions were performed in either a 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 was 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 (comprised 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, 2002), 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 from11/7 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 Ľubietová-Podlipa also contained significantly higher content of Cu when compared to those collected in Harmanec and Chvaletice. Lichens *Cladonia* cf. *novochlorophaea* and *C. pyxidata* collected from Cu mine-spoil heaps in Špania dolina and Gelnica was 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 selected 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 *Cetraria islandica*; *Asterochloris* clade A in *Cladonia mitis* and *Cladonia pleurota*, clade D in *Cladonia furcata*. On the other hand, in the fungal species *Cladonia humilis*, *C. macilenta*, *C. pyxidata*, *C. rei* and *C. 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 clade 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 occuring 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 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*, *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 correponds well to the 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, 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 *Trebouxia* "*letharii*" (Kroken and Taylor, 2000). All these taxa form a big clade, which 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), 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 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, Ľubietová-Podlipa and Gelnica when compared to 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 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 to control localities, Cu concentrations determined in lichens were much lower

when compared to 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 of 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 then that of *C. mitis* (see Table 2), *C. pyxidata* from Gelnica (Backor 16A) with Cu content 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), *C. furcata* with *C. rei*, *C. fimbriata*, etc. (clade D).

Similarly, there were not distinct differences in diversity of photobionts from localities with high and low amount of heavy metals. From the five well resolved algal lineages occurring in heavy-metal rich sites, three lineages were detected in unpolluted locality Harmanec (the same photobiont was also detected in *C. rei* from Špania dolina (Backor 23B) and *C. ochrochlora* from Harmanec (Backor 02)). Moreover, we can reject the specific influence of the Cu to photobiont community composition. In Chvaletice, where the Cu content is inconsiderable, 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 of these results: 1) the highest concentrations of heavy metals measured

in thalli of investigated lichens are not high 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*), another of them (e.g. *Cetraria islandica*, *C. aculeata*, *Cladonia 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 (Michele D. Piercey-Normore, pers. comm.). Algae from *A.* clade C are probably common on varying toxic substrates, because beside 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 other 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. *Trebouxia hypogymniae* ined. also fulfils such definition. It was identified in lichens from very varying conditions. Beside our samples from heavy metal contaminated areas, it is known also from European specimens of *Lecidea lapicida* and *L. 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 (Opanowitz 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 with 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, multiple algal genotypes

The degree of selectivity to the 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 belongs 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 another 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 (Fig. 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 *Cladonia coccifera* group (*C. coccifera*, *C. pleurota*), which associated only with *Asterochloris* clade A similar to reindeer lichens *C. arbuscula*, *C. mitis* and *C. 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 then one algal genotype 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 photobiont as well as mycobiont genotype and therefore the resulting thallus can be the 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 *Cladonia macilenta* Clad 05A and C, *C. subulata* Clad 13A and B) may attach to neighbouring 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 alga. 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 the 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 the 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 of 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 of 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 *Cladonia 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 another 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 *Diploschistes 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 r DNA sequences we detected five well resolved and other weakly supported clades within only 50 lichen samples from five 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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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	Ľubietová	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	Ľubietová	FM945362
Cladonia humilis	Clad 11 A, B	Peksa 919	Chvaletice	FM945348–9
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	FM945364–6
Cladonia mitis	Backor 01	Peksa 808	Ľubietová	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	Ľubietová	FM945371
Cladonia pyxidata	Backor 16 A, B	Peksa 791	Gelnica	FM945373–4
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	FM945355–6
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	FM945381–2
Cladonia rei	Backor 22 A, B	Peksa 809	Ľubietová	FM945386–7
Cladonia subulata	Clad 10	Peksa 926	Chvaletice	FM945379
Cladonia subulata	Clad 13 A, B	Peksa 916	Chvaletice	FM945383–4
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

Table 2. Cu content (μg/g) in selected lichen species and soils collected at five localities; H = Harmanec, SD = Špania dolina, L = Ľubietová – 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

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 analysed**
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 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 cf. *novochlorophaea*	1	1	1
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

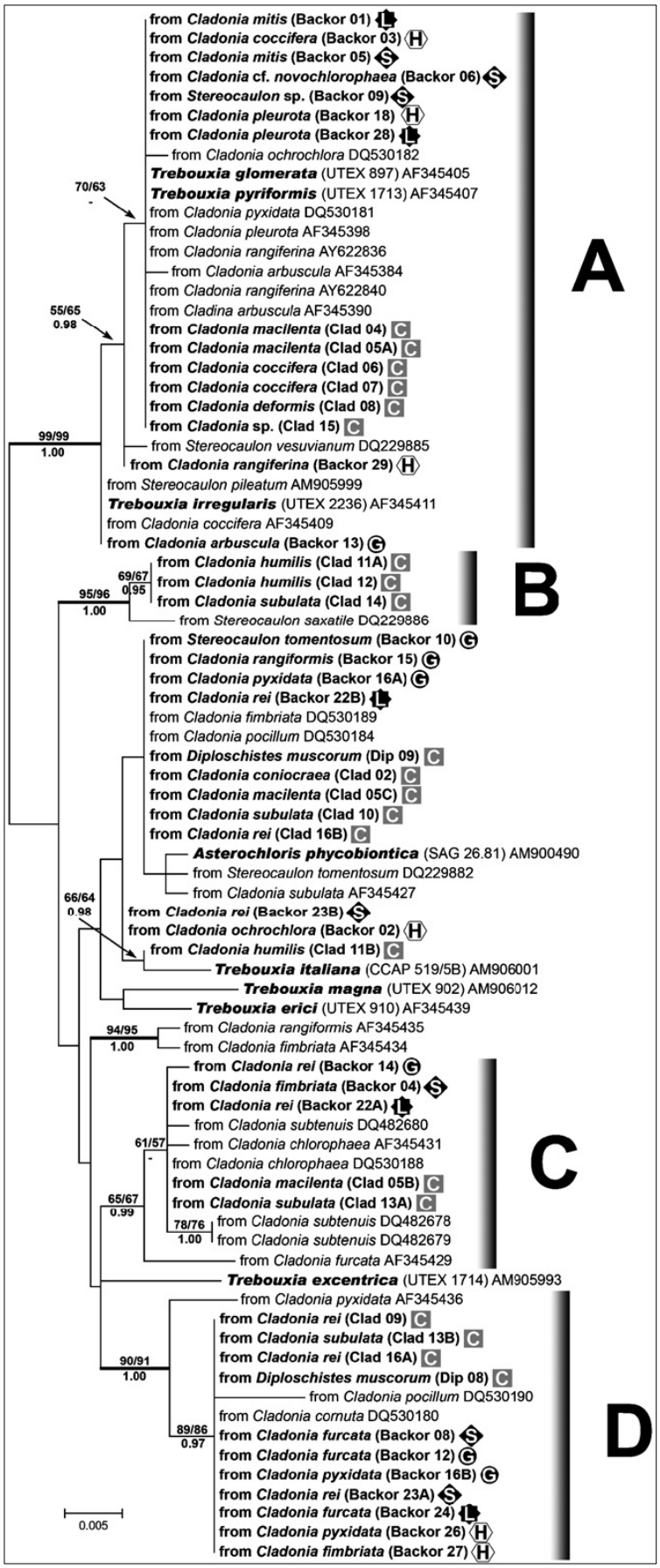

Fig. 1. ML phylogram of *Asterochloris* algae based on ITS rDNA sequences using a HKY+I+Γ model. Values at the nodes indicate statistical support estimated by three methods – maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). 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 – Ľubietová-Podlipa, S – Špania dolina). Different colors of symbols indicate degree of pollution (locality unpolluted by heavy metals are given in white, Cu mine-spoil heaps in black, and polluted locality with low Cu content in grey). Scale bar – substitutions per site.

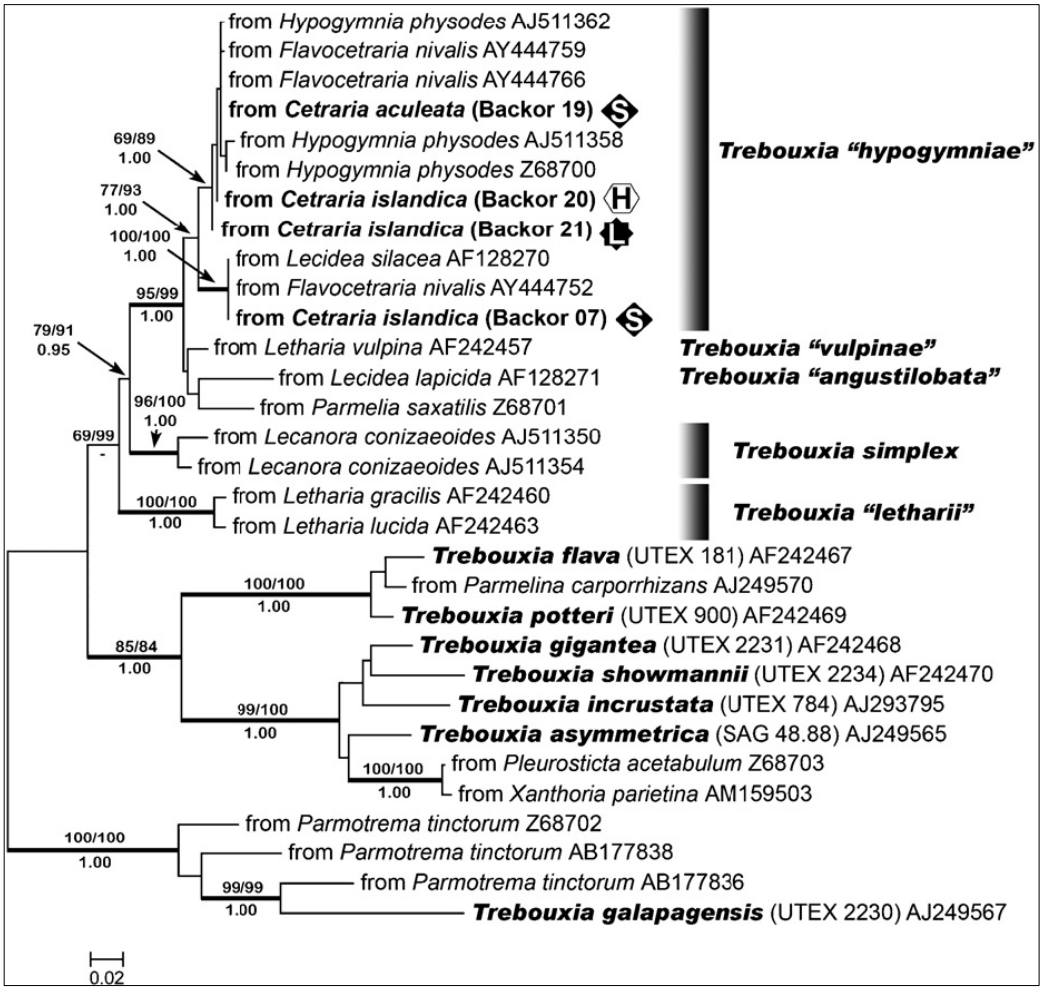

Fig. 2. ML phylogram of *Trebouxia* algae based on ITS rDNA sequences using a GTR+I+Γ model. Values at the nodes indicate statistical support estimated by three methods – maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick 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.

Paper 3

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Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae)

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Abstract

The distribution patterns of symbiotic algae are thought to be conferred mainly by their hosts, however, they may originate in algal environmental requirements as well. In lichens, predominantly terrestrial associations of fungi with algae or cyanobacteria, the ecological preferencens of photobionts have not been directly studied so far. Here, we examine the putative environmental requirements in lichenized alga *Asterochloris*, and search for the existence of ecological guilds in *Asterochloris*-associating lichens. Therefore, the presence of phylogenetic signal in several environmental traits was tested. Phylogenetic analysis based on the concatenated set of ITS rDNA and actin type I intron sequences from photobionts associated with lichens of the genera *Lepraria* and *Stereocaulon* (Stereocaulaceae, Ascomycota) revealed thirteen well-resolved clades. Photobionts from particular algal clades were found in taxonomically different but ecologically similar lichens. The rain and sun exposure was the most significant environmental factor, clearly distinguishing the *Asterochloris* lineages. The photobionts from ombrophobic and ombrophilic lichens were clustered in completely distinct clades. Moreover, two photobiont taxa were obviously differentiated based on their substrate and climatic preferences. Our study thus reveals that the photobiont, generally the subsidiary member of the symbiotic lichen association, could exhibit clear preferences for environmental factors. These preferences control the ecology of lichens and lead to the existence of specific lichen guilds.

Keywords: *Asterochloris*, ecology, *Lepraria,* lichen guilds, ombrophoby, photobiont, phylogenetic signal, symbiosis

1. Introduction

A number of aquatic as well as terrestrial algae and cyanobacteria live in various symbiotic associations. In particular, they play a role of endosymbionts in heterotrophic hosts – protists (ciliates) and invertebrates (scleractinian corals, sea anemones, sponges, green hydras) – or

they habitate the lichen thalli formed by lichen-forming fungi (ascomycetes or basidiomycetes).

In case of the corals and lichens, the nature of the symbiosis can be described as controlled parasitism whereby the host (exhabitant) actively "farms" its domesticated autotrophic partner (Ahmadjian & Jacobs 1981; Lücking *et al.* 2010; Wooldridge 2010). Within such association, a photosynthetic partner (photobiont) releases a substantial part of photosynthates to its heterotrophic partner. Furthermore, some cyanobacteria supply their host with the nitrogen fixed from the atmosphere.

The symbiotic associations exhibit a distinct measure of specificity of their symbiotic partners (the degree of taxonomic difference among partners with which an organism associates, Smith & Douglas 1987). Typically, marine invertebrates, such as reef corals and sea anemones, usually associate with unicellular dinoflagellate algae from the genus *Symbiodinium* (e.g. Mueller-Parker & Davy 2001; Coffroth & Santos 2005), although the exception to this rule was recently described by Letsch et al. (2009). Similarly, autotrophic symbionts of different freshwater protozoa and invertebrates are known to be members of various green algae (Pröschold et al. 2010). In lichen-forming fungi, many genera, or even families, were found to be exlusively associated with terrestrial green alga *Trebouxia*, or cyanobacterial genus *Nostoc* (Miadlikovska *et al.* 2006). However, the specificity of partners has been revealed as rather low at the level of species or populations. The exhabitant species can associate with multiple lineages (species) of compatible algae or cyanobacteria, and they are even able to switch between them (e.g. Friedl 1987; Ulstrup & van Oppen 2003; Guzow-Krzemínska 2006; Abrego *et al.* 2009; Bačkor *et al.* 2010). Simultaneously, particular photobiont lineage could be found in more than one species of the host, i. e. several hosts can share the same alga or cyanobacterium (e.g. Piercey-Normore & DePriest 2001; Beck *et al.* 2002; Fabricius et al. 2004; Yahr *et al.* 2004; Doering & Piercey-Normore 2009, Finney *et al.* 2010).

What are the reasons for switching between symbiotic partners? Different photobionts have been detected in the host species or communities growing in different environmental conditions. In case of the coral-alga associations, differences in irradiance and temperature have been found to affect the composition of the *Symbiodinium* community. The light-dependent distribution of individual *Symbiodinium* lineages within the coral colonies has been reported in several studies (Rowan & Knowlton 1995; Rowan *et al.* 1997; van Oppen *et al.* 2001). Sampayo *et al.* (2007) found two coral species associated with multiple symbiont profiles that showed a strong zonation with depth (irradiance). Similarly, Finney *et al.* (2010)

showed that habitat depth and geographic isolation appeared to influence the bathymetric zonation and regional distribution for most of the *Symbiodinium* species.

Interestingly, the analogical patterns have been reported from terrestrial conditions – in lichens. Cordeiro *et al.* (2005) found different lineages of *Trebouxia* algae in tropical and temperate lichens. The occurrence of different photobionts along the gradient of altitude (climate) has been reported for crustose epilithic lichens (Blaha *et al.* 2006; Muggia *et al.* 2008) as well as fruticose epiphytic lichens (Kroken & Taylor 2000). In the lichen family Physciaceae, Helms (2003) revealed the photobiont phylogeny much closer correlated with environmental factors than the phylogeny of the host fungi. According to his results, two *Trebouxia* lineages predominantly occurred in the tropics. Moreover, photobionts from basiphilous lichens growing on calcareous rocks formed a single lineage distinct from that of photobionts detected in acidophytic lichens. Interesting pattern in distribution of lichenized cyanobacteria in lichen communities associated with old-growth forests was described by Rikkinen *et al.* (2002), who found that *Nostoc* strains from epiphytic lichens were genetically separated from the strains associated with lichens growing on the ground. Such pattern forms a system of lichen guilds (the communities of lichens growing in the same habitat, sharing the same photobiont).

Thus, these findings suggest that an organism can switch its partners according to environmental conditions of its habitat. Probably, the host seeks to obtain a photobiont well adapted to local conditions because only well prospering photobiont, exhibiting maximum photosynthetic activity, can nourish its host effectively. Therefore, such exchange can increase the fitness of the host as well as of the whole association (the holobiont).

According to this hypothesis, autotrophic symbionts, generally the subsidiary members of the symbiotic associations could show their own preferences for environmental factors and influence the distribution of their hosts.

The aim of this study is to test the existence of environmental preferences in symbiotic green lichenized alga *Asterochloris*, and to verify the existence of ecological guilds in green algal lichens. We focused on the lichen genus *Lepraria* and its sister taxon *Stereocaulon* (Stereocaulaceae, Ascomycota), known for their specificity to *Asterochloris* algae (Piercey-Normore & DePriest 2001; Nelsen & Gargas 2006, 2008). The members of *Lepraria* are completely sterile, morphologically simple lichenized fungi with cosmopolitan distribution (Orange & Laundon 2009). The species are very variable in their requirements to the substrate type and climate, however, two distinct groups could be defined within the genus, based on their relationship to liquid precipitation: the ombrophiles and ombrophobes. Interestingly, the

latter strategy represents the predominant life style within *Lepraria*. Such ombrophobic species grow in fully rain-sheltered sites, often with high air humidity and low illumination where the vapor is the only available source of water (e.g. rock overhangs, some patches on tree trunks). The ability to survive under such specific conditions is likely provided by their morphological adaptation: they posses very simple thallus lacking complex structures, which is evidently very effective in the absorption of water from the air (such adaptation is known also in other lichens growing under similar conditions, e.g. *Chaenotheca*, *Chrysothrix*, *Psilolechia*).

The specific water conditions as well as the lower illumination definitely influence the photosynthesis of the symbiotic algae that is fundamental for life of the lichen. Thus, an adaptation of the photobiont seems to be necessary for the successful survival of the lichen in rain-sheltered habitats. In contrast, a life on surfaces exposed to the rain and direct sun irradiance requires tolerance of the symbionts to desiccation, temperature extremes, and high light intensities (Beckett *et al.* 2008). Therefore, we hypothesized that the ombrophilic and ombrophobic lichens should host different algal genotypes. We searched for this pattern within the genus *Lepraria* (as well as in entirely ombrophilic sister genus *Stereocaulon*) using phylogenetic analysis based on a concatenated set of ITS rDNA and actin type I intron sequences. In addition, we tested the substrate and climatic preferences of particular algal genotypes. Our results clearly indicated ecological preferences of the photobionts, suggesting the existence of lichen guilds.

2. Material and methods

2.1. Taxon sampling and determination

A total of 104 *Lepraria* s. str. and 3 *Stereocaulon* samples were collected in Europe and North America (Table S1, Supporting information). Lichen specimens were deposited in the herbaria PL (O. Peksa) and PRA (Š. Slavíková-Bayerová, Z. Palice). The sampling sites represented various habitats (diverse rock outcrops, boulder screes, forest and roadside trees, etc.) up to 2440 m above sea level (m.a.s.l).

Lichens were identified using conventional lichenological 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). Within taxa identified, variability in secondary product chemistry was detected, especially in *Lepraria caesioalba* (6 chemotypes). *Lepraria* specimens containing only atranorin and angardianic (or roccellic) acid as their main substances were denoted *Lepraria* sp., reflecting different opinions on the correct taxonomic

classification of this chemotype (Leuckert *et al.* 1995; Lohtander 1995; Tønsberg 2004; Saag 2007). We are aware of taxonomically problematic members within *Lepraria* (especially in *L. neglecta* "core group" sensu Fehrer et al. 2008). For the present study however, we accepted the distinction among the majority of the principal species based on differences in their secondary chemistry and supported partly by recent phylogenetic studies of ITS rDNA sequences (Ekman & Tønsberg 2002; Slavíková-Bayerová & Fehrer 2007; Fehrer *et al.* 2008, Nelsen & Gargas 2008).

Photobionts from 46 samples were isolated into unialgal cultures (see Table S1, Supporting information; for method of isolation and cultivation see Peksa & Škaloud 2008). Algal strains were deposited in the Culture Collection of Algae of Charles University in Prague, Czech Republic (CAUP).

2.2. DNA isolation, PCR amplification, and sequencing

Total genomic DNA was extracted from 46 algal cultures and 61 lichen thalli following the standard CTAB protocol (Doyle & Doyle 1987), with minor modifications. DNA was resuspended in sterile dH_2O and amplified by polymerase chain reaction (PCR). The ITS1-5.8S-ITS2 rDNA region was amplified using the algal-specific primer nr-SSU-1780-5' (5'-CTG CGG AAG GAT CAT TGA TTC-3'; Piercey-Normore & DePriest 2001) and a universal primer ITS4-3' (5'-TCC TCC GCT TAT TGA TAT GC-3'; White *et al.* 1990). Actin type I locus (1 complete exon and two introns located at codon positions 206 and 248; Weber & Kabsch 1994) was amplified using the algal-specific primers ActinF2 Astero (5'-AGC GCG GGT ACA GCT TCA C-3') and ActinR2 Astero (5'-CAG CAC TTC AGG GCA GCG GAA-3'; Skaloud & Peksa 2010). All PCR reactions were performed in 20 µl reaction volumes (15.1 µl sterile Milli-Q Water, 2 µl 10' PCR buffer (Sigma), 0.4 µl dNTP (10 µM), 0.25 µl of primers (25 pmol ml^{-1}), 0.5 µl Red Taq DNA Polymerase (Sigma) (1U ml^{-1}), 0.5 µl of $MgCl_2$ (25 mM), 1 µl of DNA (not quantified). PCR and cycle-sequencing reactions were performed in either a XP thermal cycler (Bioer) or a Touchgene gradient cycler (Techne). PCR amplification of the algal ITS began with an initial denaturation at 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 a 1% agarose gel stained with ethidium bromide and purified using either the JetQuick PCR Purification Kit (Genomed) or the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocols. The

purified amplification products were sequenced with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730XL) using the PCR primers from Macrogen Corp. in Seoul, Korea. Sequencing readings were assembled and edited using SeqAssem programme (SequentiX Software).

2.3. Sequence alignment and DNA analyses

Sequences were initially aligned using MUSCLE alignment software (Edgar 2004). Photobiont sequences deposited in GenBank (26 seq. from *Lepraria* and 14 seq. from *Stereocaulon* photobionts) were acquired and included in the alignment. In *Lepraria*, we included only those GenBank photobiont sequences acquired from lichens determined at the species level. After deleting identical sequences obtained from the same lichen taxa, the resulting concatenated alignment comprised 64 sequences (incl. 64 ITS rDNA and 38 actin type I locus sequences; missing actin data were replaced with question marks according to Rannala & Yang 2003). ITS sequences were aligned on the basis of their rRNA secondary structure information, the alignment of actin I locus sequences has been improved through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP v. 1.2 alpha 4 (Löytynoja & Milinkovitch 2001). For detailed information about alignment improvement see Skaloud & Peksa (2010). The resulting concatenated alignment had a length of 1173 characters (ITS, 514; actin, 659; available from the second author upon request). The congruence of data partitions that allows their merging into a concatenated alignment has been previously justified by inspecting bootstrap scores above 70% resulting from separate ML and MP analyses of the ITS and actin data set (Skaloud & Peksa 2010).

Bayesian inference (BI) was performed with MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). The alignment was divided into six region partitions (ITS1, ITS2, 5.8S rRNA, actin intron 206, actin intron 248, actin exon), and for each partition the most appropriate substitution model was estimated using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004). Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach (MCMC). Two parallel MCMC runs were carried out for 3 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. The stationary distribution of the runs was confirmed by checking average standard deviations of split frequencies between the two analyses, which approached zero. Convergence of the two cold chains was checked and burn-in was determined using the "sump" command.

Bootstrap analyses were performed by maximum likelihood (ML) and weighted parsimony

(wMP) criteria using PAUP*, version 4.0b10 (Swofford 2002). ML analyses (100 replicates) consisted of heuristic searches using the neighbor-joining tree as the starting tree, Tree bisection reconnection swapping algorithm, and number of rearrangements limited to 10,000. The analysis was conducted using unpartitioned alignment with GTR+Γ+I model. The wMP analyses (1,000 replicates) 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. Bootstrap percentages and posterior probabilities were interpreted as weak (less than 50%), moderate (50–94% for BI; 50–79% for ML and MP) or high (more than 94% for BI; more than 79% for ML and MP).

2.4. Analyses of ecological relatioships

The following environmental data were collected for each lichen sample: exposure to rain (exposed/sheltered), altitude (in metres a.s.l.), and type of substrate (wood-bark; basic type of bedrock – basalt, gneiss, granite, sandstone, shale and serpentine – coded as a set of dummy variables).

To analyze possible ecological preferences of particular photobiont lineages, we conducted three different tests for the existence of phylogenetic signal in our data (according to Blomberg *et al.* 2003, the phylogenetic signal is the tendency for related species to resemble each other). All calculations were performed in the program R, ver. 2.9.2 (The R Foundation for Statistical Computing 2009, http://www.r-project.org/). First, we tested the phylogenetic signal using Pagel's lambda (Pagel 1999). This test uses a tree transformation parameter that has the effect of gradually eliminating phylogenetic structure. The maximum likelihood optimization of Lambda value was performed using the 'fitDiscrete' or 'fitContinuous' functions of the Geiger package (Harmon *et al.* 2008). To test for the existence of phylogenetic signal in the dataset, we compared the negative log likelihoods obtained from a tree without phylogenetic signal and the original topology, using likelihood ratio test. Second, the phylogenetic signal was tested using K statistic (Blomberg *et al.* 2003). This statictic quantifies the phylogenetic signal by estimating the accuracy of the original phylogeny to describe the variance-covariance pattern observed in the datatest. The K value and randomization test were calculated by 'Kcalc' and 'phylosignal' functions of the Picante package (Kembel *et al.* 2009).

Finally, the existence of phylogenetic signal was tested by searching for significant ecological similarity in selected set of closely related organisms (organisms with short genetic

distance), using our simple customized R script (see Appendix S1, Supporting information). The ecological similarity was evaluated as the sum of Euclidean distances of the environmental data. The small value of sum of Euclidean distances signified high ecological similarity of the examined samples (if all samples had the same value of ecological factor, the sum of Euclidean distances would be zero).The genetic distances were calculated using Kimura 2 parameter substitution model on the concatenated data using MEGA4. The distances of environmental data were calculated using PAST, ver. 1.90 (Hammer *et al.* 2001) using Euclidean distances.

First, we specified the genetic distance which delimit closely related strains by analyzing histogram of frequency distribution of pairwise genetic distances. The apparent gap in the histogram around the distance of 0.04 led us to select this value to define the closely related strains belonging to one, or seldom two, phylogenetic lineages as revealed by Bayesian phylogenetic analysis (Fig. S1, Supporting information). Next, the sum of Euclidean distances of environmental data was calculated for the set of photobiont pairs whose genetic distances were lower than the selected value delimiting the closely related strains. Finally, the existence of phylogenetic signal (i.e. significant ecological similarity in closely related strains) was tested by non-parametric permutation of all photobiont pairs (100,000 replicates).

Since all above-mentioned tests demonstrated the existence of phylogenetis structure in our data, we used the program BayesTraits (Pagel & Meade 2006), which combines Bayesian and maximum likelihood based approaches, to test the contingency of character evolution. First, the evolution of ombrophoby was reconstructed using BayesMultiState in an ML framework over all common ancestors (using the 'addNnode' command). We adjusted the 'Mltries' parameter to 100 to increase the number of optimalisation attemps. The BayesTraits output was mapped onto the reference tree with TreeGradients v1.03 (Verbruggen 2009). This program plots ancestral state probabilities on a phylogenetic tree as colors along a color gradient. Second, the ancestral state probabilities of selected environmental parameters (types of substrate) were calculated for the most common ancestors of all highly-supported clades.

Some relationships among ecological factors and photobionts were also examined using descriptive statistics (box plots) in Statistica ver. 8 – Statsoft, Inc. (Hill & Lewicki 2007) and Principal Component Analysis in Canoco for Windows, ver. 4.5 (ter Braak & Šmilauer 1998).

3. Results

3.1. Phylogenetic analysis

Data on length, variability, and base composition of the molecular markers, as well as the evolutionary models estimated for each partition can be found in Table S2, Supporting information. Substantial differences were revealed in the sequence variability and estimated substitution models among the individual partitions. Whereas the whole ITS rDNA dataset comprised only 48 parsimony informative sites, both actin intron partitions were quite rich in variable sites (112 and 162 parsimony informative sites, respectively).

The concatenated alignment contained genetic data for 147 lichen photobionts, including 130 samples of photobionts from *Lepraria* and 17 samples of *Stereocaulon* photobionts.

The phylogram resulting from Bayesian analysis of ITS rDNA and actin type I sequences is presented in Fig. 1. The analyzed lichen photobionts clustered in 13 well-supported clades. Three of these clades could be assigned to the formally described phenotypic species: *Asterochloris phycobiontica* (clade A1), *Asterochloris glomerata* (clade A12) and *Asterochloris irregularis* (clade A13). Although clades A2, A12 and A13 achieved low levels of statistical support in the current analysis, they all were statistically significantly supported by the phylogenetic analysis in our previous study of *Asterochloris* algae (Skaloud & Peksa 2010). The low statistical significance in the current analysis was very probably caused by the absence of actin type I sequences in several strains from these three clades.

3.2. Specificity in lichen associations

We analyzed photobionts from 16 *Lepraria* and 8 *Stereocaulon* phenotypic species (excluding several incompletely determined samples of *Stereocaulon*). We are aware that the lichen identification based on their morphological and chemical characters did probably not reveal the genuine diversity of the lichen-forming fungi, however, it was sufficient to provide rough information on the specificity in lichen associations.

Each lichen specimen contained only one specific photobiont genotype. The degree of specificity varied among both photobiont lineages and fungal species. Each algal lineage was shared by at least two (clades A2, A4, A6, A8, A9), and up to eight (clade A7) fungal species (see Fig. 1). Simultaneously, the majority of fungal species were associated with a polyphyletic assemblage of algae from several clades, indicating the ability of the fungi to switch their photobionts (e.g. different samples of *Lepraria alpina* with clades A1, A2, A3, A9). Only three *Lepraria* species (from those represented by at least 10 specimens) were found to be associated with one individual algal clade with an apparently higher frequency:

Lepraria borealis (clade A5), *Lepraria lobificans* (clade A7) and *Lepraria rigidula* (clade A10).

We did not observe the sharing of algal lineages between the two analyzed fungal genera, i.e. the members of *Lepraria* associated with different algal clades than the members of *Stereocaulon*.

3.3. Environmental preferences of photobionts

To analyze possible environmental preferences of photobionts, the presence of phylogenetic signal in three environmental traits was examined (exposure to rain, altitude, substrate type). For each trait, Pagel's lambda and K statistics were calculated to show influence of the inferred phylogeny (Fig. 1) on trait variance across photobiont strains. Both methods revealed significant phylogenetic signal in all traits (Table 1).

Moreover, we devised additional method to test the existence of phylogenetic signal by searching for significant ecological similarity in closely related strains (for details of the method see Material and Methods). In the presence of phylogenetic signal, the photobionts having short genetic distances from each other (i.e. closely related) should be ecologically similar. Accordingly, environmental preferences of photobionts were detected by comparing the similarity of environmental data of genetically close photobiont pairs to that of genetically distant pairs (the value of genetic distance distinguishing closely related and distant algal strains was identified at 0.04). We detected significant ecological similarity in the set of photobiont pairs with short genetic distances (Table 1).

All environmental traits showed significant phylogenetic signal, whichever method was employed. In other words, closely related photobionts tended to be similar in each environmental characteristic: they occured in lichens growing in habitats characterized by similar water regime (rain-exposed or rain-sheltered surfaces), similar climate (limited range of altitudes) and similar type of substrate.

To illustrate the ecological preferences of *Lepraria* photobionts more clearly, we mapped the evolution of a selected ecological character the relationship to precipitation - onto the phylogenetic tree (Fig. 2). The relationship of the lichen to liquid water was chosen as an ideal character for the evolutionary mapping because it can have only two aspects: a lichen grows on exposed or sheltered surface, i.e. it is either ombrophilic or ombrophobic. Moreover, owing to the broad knowledge of this character in most lichens, we were able to assign not only our samples but also those obtained from GenBank. In total, we had 92 photobiont sequences from ombrophilic lichens and 55 sequences from ombrophobic lichens. The result

of character mapping clearly showed the prevailing presence of clades A4, A6, A7 and A10 in ombrophobic *Lepraria* species (with ancestral probabilities for ombrophoby 0.99, 0.99, 0.88 and 1.00, respectively). The other clades (including all weakly supported lineages) were completely associated with ombrophilic lichens.

Among all samples, two interesting exceptions have occured, indicating the sensitivity of photobiont to the water and light regime in its (micro)habitat. First, the sample OP186 (clade A3) was labeled as ombrophilic (see Fig. 2) although it originated from the common ombrophobic species *Lepraria rigidula*. However, this specimen occurred abnormally on bryophytes covering the rain-exposed edge of a rock. Interestingly, the ombrophilic character of this sample coincided accurately with the nature of all other samples of the clade A3. Second, the sample OP526 from the ombrophilic *Lepraria caesioalba* (clade A4) was collected from the upper surface of a boulder sheltered by deciduous oak tree. The sample was labeled as ombrophilic because the thallus was not fully sheltered against precipitation (especially in the winter). However, it clustered with strictly ombrophobic samples in the clade A4. Probably, the specific water and light conditions under the tree-top corresponded rather to rain/sun-sheltered habitats. In both cases, the photobiont type correlated with the environmental conditions rather than with the mycobiont nature.

In addition to water conditions, some clades were found to be completely dissimilar in their altitude and substrate preferences. The algae of the clade A1 (*A. phycobiontica*) occurred predominantly in regions with altitudes of about 1500 m , whereas members of the clade A5 were found mostly in areas around 450 m a.s.l. (Fig. 3). Moreover, both these clades were correlated with different substrates, as shown by the principal component analysis (Fig. 4). Both were found in ombrophilic lichens collected from bryophytes, soil or directly from rock surface, however, photobionts of the clade A1 were contained rather in lichens growing in habitats of acidic siliceous rocks (rocks and screes from granite or gneiss), while lichens housing photobionts of the clade A5 preferred SiO_2-poor rocks (shales or basalts). In addition, the clade A10 was most often associated with ombrophobic lichens growing on the bark of broadleaf trees.

To test the substrate preferences of individual clades, we modelled the evolution of the preference for each substrate and inferred ancestral probabilities, for each of the thirteen well-supported clades (Table 2). According to the inferred ancestral probabilities, some lineages exhibited significant substrate preferences towards shale, wood, and basalt, whereas for granite-gneiss, serpentine, and sandstone no apparent preferences have been detected along the entire phylogenetic tree. The analysis confirmed the high affinity of the clades A5 and

A10 to shale and wood, respectively. Conversely, negative substrate preferences were detected for the clades A2 (shale and wood), A8 (shale) and A11 (wood).

4. Discussion

Lepraria and *Stereocaulon* represent two closely related genera of lichen-forming ascomycetes (Ekman & Tønsberg 2002; Myllys *et al.* 2005). We found that these fungi associate with green-algal photobionts from the genus *Asterochloris*. In approx. 25 lichen morphospecies, thirteen well-supported *Asterochloris* clades and several additional weakly-supported lineages were revealed, indicating relatively high diversity of photobionts in *Lepraria* and *Stereocaulon* (some of the weakly-supported lineages were inferred with a high statistical support in previous studies by Nelsen & Gargas 2008 and Skaloud & Peksa 2010).

The observed variation in lichen associations (switching of the partners) suggests that the specificity between symbionts is not the only determinant of the composition of individual association (the lichen thallus). It can be further influenced by the availability (occurrence) of compatible partners in relation to the environmental conditions at the locality.

Each species of alga or cyanobacterium presents with specific optimum for its photosynthetic as well as other metabolic activities. An unsuitable light, water or climatic regime may cause low fitness of the photobiont leading to its very low abundance or even absence in certain habitat. The mycobiont, as an exhabitant, shelters its partner against the harmful UV radiation, it can partly regulate the water content within the thalli (Honegger 2006, 2009), or it can protect the photobiont against the direct influence of substrate pH (Mollenhauer 1997). Nevertheless, the potential of the lichen fungi to protect their photobionts are necessarily limited because the lichen is actually poikilohydric system strongly dependent on the local climatic regime. Furthermore, the ecology of the algae or cyanobacteria has their own history, preceding the lichenization event. Some photobionts are still recruited from free-living forms (Wirtz *et al.* 2003), other such as Trebouxia were only found in form of small free-living colonies (Tschermak-Woess 1978; Bubrick *et al.* 1984; Mukhtar *et al.* 1994; Sanders 2005) whose survival is probably strongly dependent on suitable local conditions.

Similarly to photobionts, the individual fungal species are adapted to specific environmental conditions. Therefore, only the associations composed from partners that are both thriving at given habitat can successfully survive.

Our research revealed that particular *Asterochloris* lineages were contained in taxonomically different but ecologically similar lichens. Especially, the exposure of the lichen

to rain and sun was found to be the crucial factor for distinguishing the *Asterochloris* lineages. The photobionts obtained from the ombrophobic lichens were genetically distinct from those obtained from the ombrophilic lichens. Clades A4, A6 and A10 were contained exclusively in *Lepraria* species growing on rain/sun-sheltered surfaces, such as vertical or overhanging rock walls and tree trunks. Conversely, the majority of other clades were associated exclusively with the ombrophilic *Lepraria* and *Stereocaulon* species growing in rain/sun-exposed situations. Thus, the dissimilarity in environmental conditions – different water, light and temperature regime – caused the distribution patterns of individual photobiont lineages.

The exceptions to this general scheme were found, especially in the clade A7, containing photobionts from lichens exhibiting both water-seeking and -avoiding types of life strategies (although the ombrophobic preference was predominant here). The dissembling nature of this clade corresponds with its extensive ecological plasticity very well; it was found to associate with a great number of fungal species growing in diverse water and light conditions, in a broad range of altitudes, and on various substrates (from base-rich to acidic substrates). Clade A7 confirms the existence of photobionts exhibiting very wide ecology. Such euryecious species were observed also by other authors (e.g. Guzow-Krzemińska 2006; Bačkor *et al.* 2010).

A number of *Asterochloris* clades were markedly tolerant to various climatic conditions and substrates (A2, A3, A10, A11). However, at least two lineages were revealed to be distinguishable based on these ecological characteristics: the clades A1 and A5 (both from ombrophilic lichens). The photobionts from the clade A1 were detected in lichens growing predominantly on siliceous rocks at altitudes above 1000 m (the lower border of mountain belt with cold and humid climate). Conversely, the clade A5 was characteristic for lichens growing predominantly on naked soils or on bryophytes in fissures of SiO_2-poor rocks (shales, basalts) at low altitudes. Correspondingly to these variations in ecology, the clades A1 and A5 differ in their associated fungi (if we omit the very common but taxonomically questionable species *Lepraria caesioalba*). Clade A1 (*Asterochloris phycobiontica*) were detected predominantly in mountain species *L. alpina* and *L. neglecta*. Moreover, *A. phycobiontica* was first isolated and described from the lichen *Anzina carneonivea* (Tschermak-Woess 1980) which is a typical psychrophilous species (cf. Palice 1999). In contrast, the clade A5 was found in *Lepraria borealis* and *L. nylanderiana*, and additionally in lowland specimens of *Cladonia foliacea*, *C. humilis* and *C. subulata* (Bačkor *et al.* 2010; Skaloud & Peksa 2010). *C. foliacea*, *C. humilis* and *L. nylanderiana* represent species typical for warm localities in lowland, colline and submontane regions.

Our observations imply that the distribution of photobionts is in some respects independent of the particular mycobiont species, being much more accorded to specific conditions and lichen communities. This fact leads us to propose the existence of ecological guilds in lichens containing green algae (a guild of ombrophobic acidophilous lichens, a guild of ombrophilic mountain lichens from siliceous rocks and a guild of ombrophilic lowland lichens from SiO_2-poor substrates). We expect that this hypothesis will be confirmed in future studies examining large photobiont inventories of lichen communities growing in climatically and/or geologically different biotopes.

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Table 1. Statistics for randomization tests showing the significance of phylogenetic signal for three environmental traits investigated. For each trait, Pagel's lambda, K statistics, and ecological similarity among closely related strains (our method) were calculated to show influence of inferred phylogeny on trait variance across *Asterochloris* strains. Lambda values could vary from 0 (no influence of phylogeny) to 1 (strong phylogenetic influence). Likelihood ratio indicates comparison of the log-likelihoods of a model with the maximum likelihood estimate of lambda for a given trait to the log-likelihood of a model where lambda was set to zero. The K values indicate how closely is the species trait correlates to its phylogeny, as expected under Brownian motion (higher K-values mean better correlation). Ecological similarity was tested in the set of photobiont pairs with short genetic distances (lower than 0.04).

	Pagel's lambda			K statistics		Ecological similarity
Trait	λ	Likelihood ratio	p-value	K value	p-value	p-value
Exposure to rain	0.946	1.53	< 0.0001	0.2126	0.001	< 0.0001
Altitude	0.045	1.01	< 0.0001	0.0832	0.005	< 0.0001
Substrate type	0.652	1.05	0.0011	0.1168	0.002	< 0.0001

Table 2. Ancestral state probabilities of the substrate preferences for particular photobiont clades. Values with clear positive (**!**) or negative (**∗**) probabilities are given in bold.

clade	shale	wood-bark	basalt	granite-gneiss	serpentine	sandstone
A1	0.21	0.15	0.38	0.50	0.50	0.50
A2	**0.06 ***	**0.02 ***	0.57	0.50	0.50	0.50
A3	0.81	0.26	0.49	0.50	0.50	0.50
A4	0.31	0.89	0.44	0.50	0.50	0.50
A5	**0.92 !**	0.23	0.54	0.50	0.50	0.50
A6	0.40	0.31	0.48	0.50	0.50	0.50
A7	0.19	0.13	0.45	0.50	0.50	0.50
A8	**0.03 ***	0.69	0.11	0.50	0.50	0.49
A9	0.35	0.20	0.48	0.50	0.50	0.50
A10	0.17	**1.00 !**	0.69	0.50	0.50	0.50
A11	0.37	**0.04 ***	0.26	0.50	0.50	0.50
A12	0.40	0.30	0.48	0.50	0.50	0.50
A13	0.42	0.33	0.49	0.50	0.50	0.50

Footnote: the substrates with nearly similar character – the siliceaous rocks *granite* and *gneiss* as well as *wood* and *bark* – were used as combined variables in the analysis.

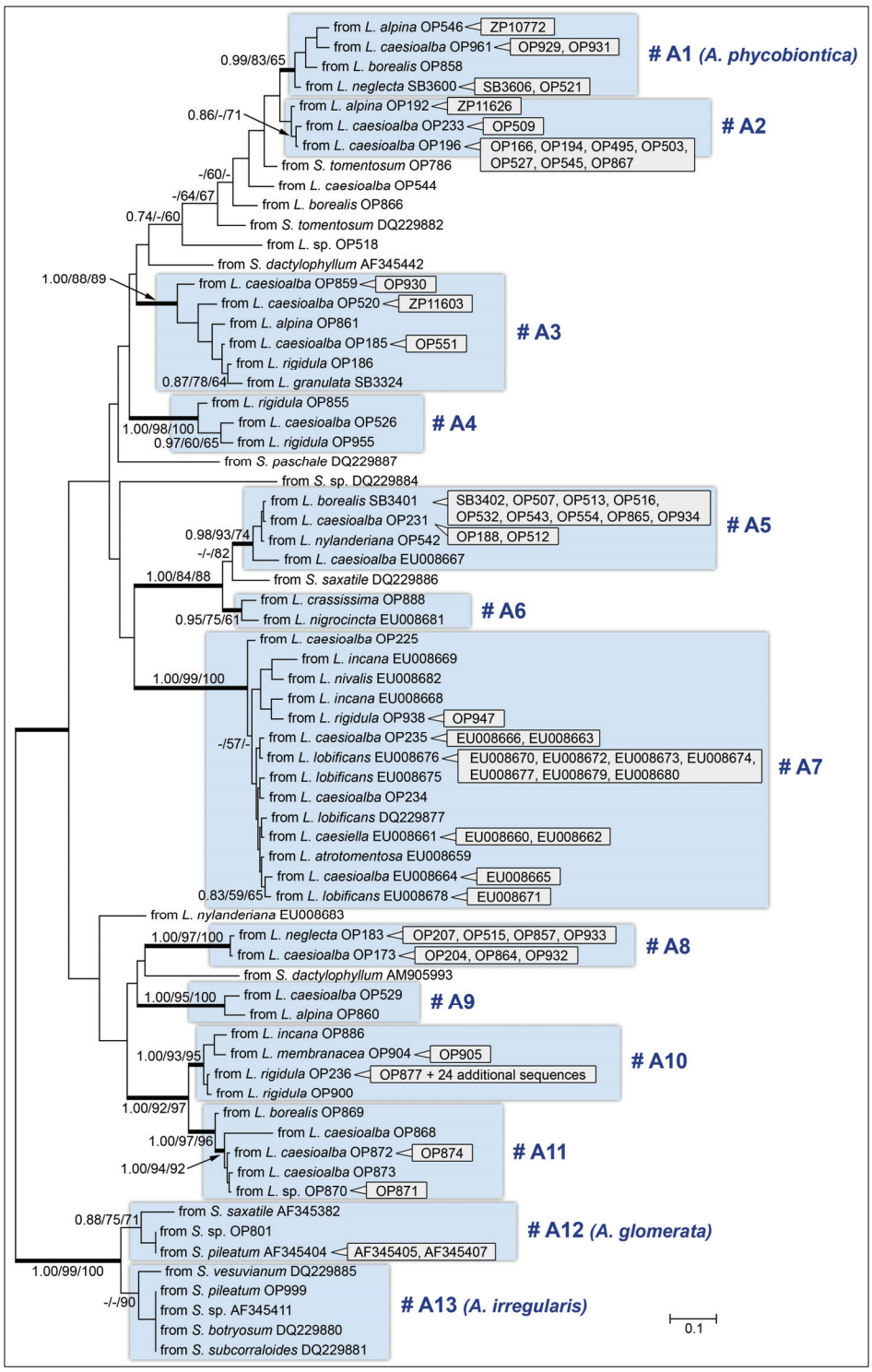

Fig. 1. Unrooted BI analysis of *Asterochloris* photobionts based on the combined ITS + actin dataset. The analysis used a HKY+I model for ITS1 and ITS2, F81 model for 5.8 rRNA partition, a HKY+G model for the actin-intron 206, GTR+G model for the actin-intron 248, and K80+I model for the actin-exon partition. Values at the nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), maximum likelihood bootstrap (middle) and maximum parsimony bootstrap (right). Support values are displayed only for nodes with BI/ML/MP supports of ≥0.70/50/50. Thick branches represent nodes receiving posterior probability ≥0.95. The affiliation of strains to the thirteen lineages is indicated (the clade labelling does not correspond to that in our previous study; see Table S1, Supporting information). The additional, identical photobiont sequences are shown in gray boxes to the right of the sequence used for the analysis. Our samples codes correspond to collection numbers (see Table S1, Supporting information): OP – Peksa, SB – Bayerová, ZP – Palice; the sequences obtained from GenBank are coded by Accession numbers. Scale bar – expected number of substitutions per site.

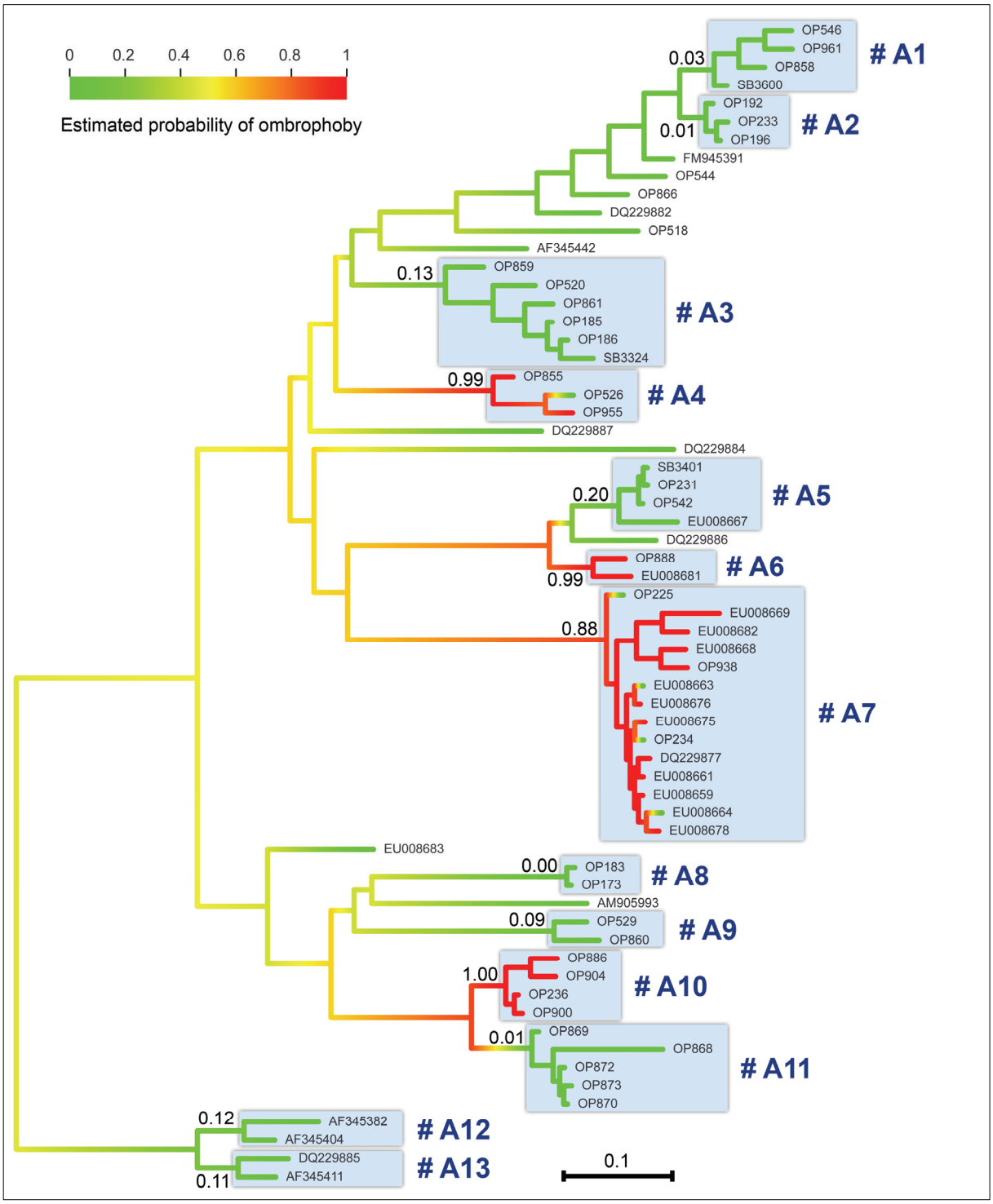

Fig. 2. The evolution of the selected ecological character – the ombrophoby (ombrophily) of lichens – mapped onto the photobiont phylogenetic tree. Colors are used to visualize estimated probabilities of the presence of ombrophoby along the phylogenetic tree. Red indicates a high probability of ombrophoby, whereas green denotes a low probability of ombrophobic preference. The estimated probabilities for ombrophoby are indicated for ancestors of each significantly supported clade (see Fig. 1). The topology of the tree corresponds completely to the topology of the phylogenetic tree in Fig. 1.

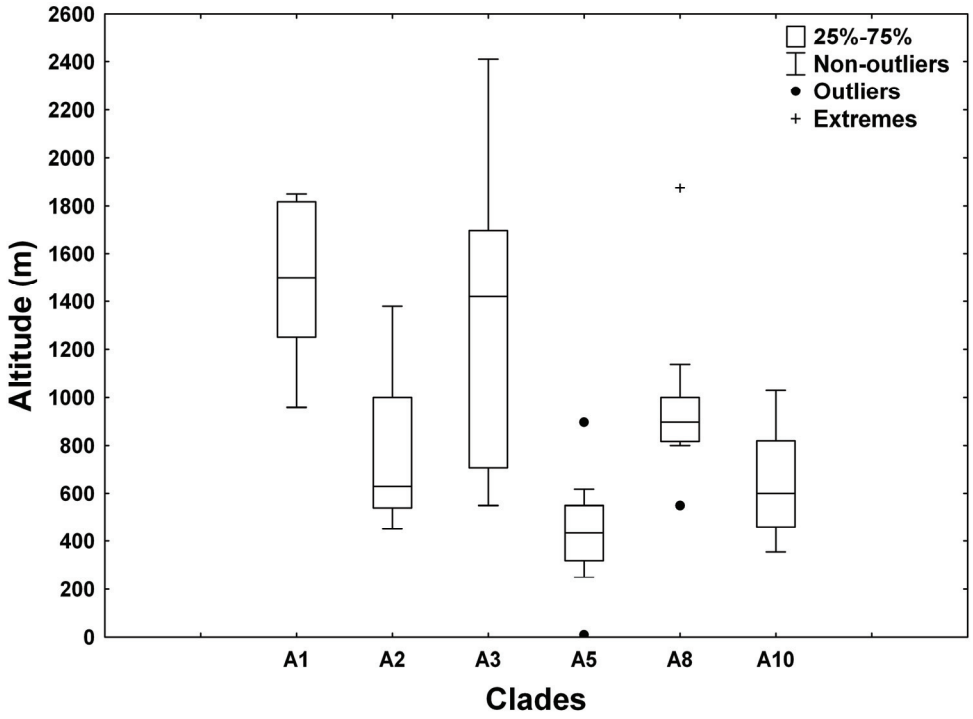

Fig. 3. Differences in the distribution of selected *Asterochloris* clades along the altitudinal gradient. Box and whisker plots are based on altitudinal data from six clades represented by at least 10 samples. All samples were collected in similar latitudes (43–51° north) in Europe. The approximate upper borders of vertical vegetation belts for central Europe are as follows: 200 m a.s.l.– lowland, 600 m – colline, 1000 m – submontane, 1400 m – montane, 1800 m – subalpine, 2400 m – alpine.

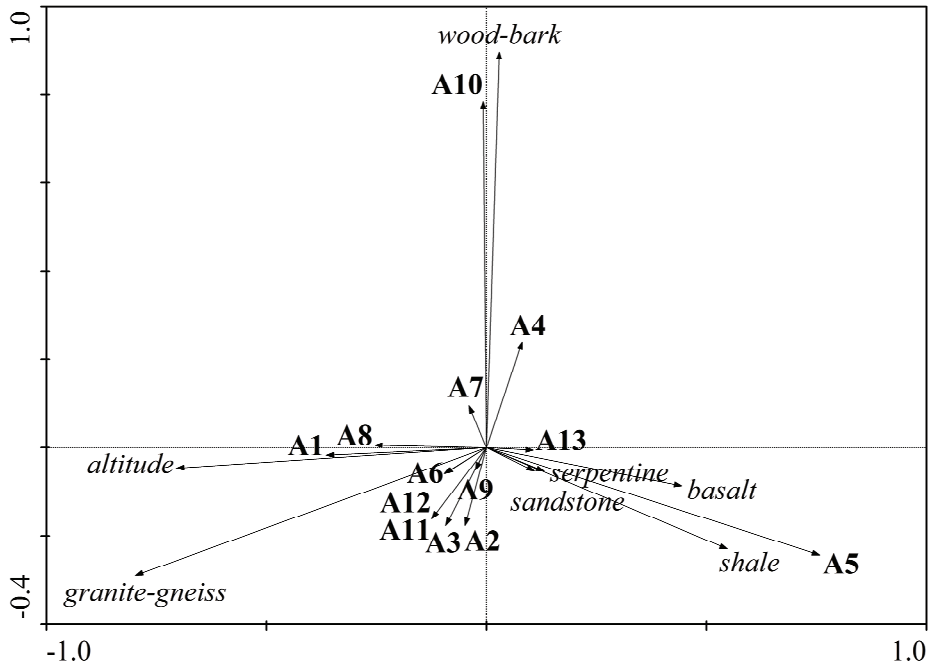

Fig. 4. The distribution of the *Asterochloris* clades (A1–A13) in relation to environmental factors: altitude and type of substrate. Ordination plot of the principal component analysis (PCA) based on 82 samples of *Lepraria* and *Stereocaulon* with complete environmental data. Note: the substrates with nearly similar character – the siliceaous rocks *granite* and *gneiss* as well as *wood* and *bark* – were used as combined variables in the analysis.

Supporting Information Table S1. List of samples used in the study with GenBank accession numbers. Chemotype – sensu Leuckert et al. (1995). Collection numbers – lichen specimens were deposited in the herbaria PL (The West Bohemian Museum in Pilsen; O. Peksa) and PRA (The Institute of Botany of the Academy of Science of the Czech Republic.; Š. Slavíková-Bayerová, Z. Palice). Culture – sequence was obtained from algal culture deposited in culture collection of O. Peksa and CAUP culture collection (http://botany.natur.cuni.cz/algo/caup). References:

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Fungal taxon	Chemotype	Origin	Collection number	Culture	GenBank accession		clade labelling sensu Skaloud & Peksa 2010
					ITS	actin	
clade A1							**clade 15 (*A. phycobiontica*)**
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Peksa 546	*	FN556023	-	
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Norway	Palice 10772	*	as FN556023	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 961	*	FN556024	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 929		as FN556024	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 931		as FN556024	-	
Lepraria neglecta (Nyl.) Lettau		Ukraine	Bayerová 3600	*	AM906013	AM906044	
Lepraria neglecta (Nyl.) Lettau		Ukraine	Bayerová 3606	*	AM900941	AM906043	
Lepraria neglecta (Nyl.) Lettau		Slovakia	Peksa 521	*	as AM900941	-	
Lepraria sp.		Austria	Peksa 858		FN556025	-	
clade A2							**clade 16**
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Peksa 192	*	AM906010	AM906039	
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Czech Republic	Palice 11626		as AM906010	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 233	*	AM906006	AM906035	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 509	*	as AM906006	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 3	Czech Republic	Peksa 196	*	AM906007	AM906036	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 166	*	AM906008	AM906037	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 194	*	AM906009	AM906038	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 545	*	as AM906009	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 503	*	as AM906009	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 495	*	as AM906009	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 527	*	as AM906009	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 3	USA, California	Peksa 867		as AM906009	-	
clade A3							**clade 14**
Lepraria alpina		Spain	Peksa 861		FN556026	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 185	*	FM955666	FM955670	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 551	*	FM955667	FM955671	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Slovakia	Peksa 520	*	FN556027	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Palice 11603		as FN556027	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Spain	Peksa 859		FN556028	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 930		as FN556028	-	
Lepraria granulata Slavíková-Bayerová		Bulgaria	Bayerová 3324		FN556029	-	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 186	*	AM905992	AM906017	

clade A4							**clade 17**
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 526	*	FN556030	-	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 855		FN556031	FN556047	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 955		FN556032	-	
clade A5							**clade 10**
Lepraria borealis Lohtander & Tønsberg		Bulgaria	Bayerová 3401	*	AM900492	AM906045	
Lepraria borealis Lohtander & Tønsberg		Bulgaria	Bayerová 3402	*	AM906015	AM906048	
Lepraria borealis Lohtander & Tønsberg		Sweden	Peksa 554	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 532	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 513	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 507	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Slovakia	Peksa 516	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 543	*	as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 865		as AM906015	-	
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 934		as AM906015	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 231	*	AM906014	AM906047	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 188	*	as AM906014	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 512	*	as AM906014	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon		Spain	Sipman 45330		EU008667	-	
Lepraria nylanderiana Kümmerl. & Leuckert		Czech Republic	Peksa 542	*	AM900493	AM906046	
Stereocaulon saxatile H.Magn.		USA, Alaska	Talbot KIS 187		DQ229886	DQ229897	
clade A6							**clade 18**
Lepraria crassissima (Hue) Lettau		Czech Republic	Peksa 888		FN556033	-	
Lepraria nigrocincta Diederich, Sérus. & Aptroot		Costa Rica, San José	Nelsen 3637b		EU008681	EU008710	
clade A7							**clade 11**
Lepraria atrotomentosa Orange & Wolseley		China, Yunnan	Nelsen 2598		EU008659	EU008693	
Lepraria caesiella R.C. Harris		USA, Massachusetts	Nelsen 3962		EU008661	EU008694	
Lepraria caesiella R.C. Harris		USA, Wisconsin	Nelsen 3953		EU008660	-	
Lepraria caesiella R.C. Harris		USA, Pennsylvania	Nelsen 3968		EU008662	EU008695	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Romania	Peksa 225	*	AM905996	AM906022	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 3	Slovakia	Peksa 234	*	AM905994	AM906020	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 2	Slovakia	Peksa 235	*	AM905995	AM906021	
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3965		EU008663	EU008696	
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3969		EU008666	EU008699	
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3966		EU008664	EU008697	
Lepraria caesioalba (de Lesd.) J.R.Laundon		USA, Pennsylvania	Nelsen 3967		EU008665	EU008698	
Lepraria incana (L.) Ach.		USA, Virginia	Nelsen 3972		EU008669	-	
Lepraria incana (L.) Ach.		USA, Maryland	Nelsen 3971		EU008668	-	

Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3974		DQ229877	DQ229898	
Lepraria lobificans Nyl.		USA, Wisconsin	Neslen 3960		EU008675	EU008704	
Lepraria lobificans Nyl.		USA, Massachusetts	Nelsen 3961		EU008676	EU008705	
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3950		EU008670	EU008700	
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3956		EU008672	-	
Lepraria lobificans Nyl.		USA, Tennessee	Nelsen 3958		EU008673	EU008702	
Lepraria lobificans Nyl.		USA, Indiana	Nelsen 3957		EU008674	EU008703	
Lepraria lobificans Nyl.		USA, West Virgina	Nelsen 3970		EU008677	EU008706	
Lepraria lobificans Nyl.		USA, Michigan	Nelsen 3976		EU008679	EU008708	
Lepraria lobificans Nyl.		China, Yunnan	Nelsen 2590		EU008680	EU008709	
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3973		EU008678	EU008707	
Lepraria lobificans Nyl.		USA, Wisconsin	Nelsen 3951		EU008671	EU008701	
Lepraria nivalis J.R.Laundon		Canada, Quebec	Olszewski 5409o		EU008682	-	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 938		FN556034	-	
Lepraria rigidula (de Lesd.) Tønsberg		Slovakia	Peksa 947		as FN556034	-	
clade A8							**clade 6**
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 173	*	AM906003	AM906032	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 204	*	AM906004	AM906033	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 932		as AM906004	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 864		as AM906004	-	
Lepraria neglecta (Nyl.) Lettau		Czech Republic	Peksa 183	*	AM906002	AM906031	
Lepraria neglecta (Nyl.) Lettau		Czech Republic	Peksa 207	*	AM906005	AM906034	
Lepraria neglecta (Nyl.) Lettau		Slovakia	Peksa 515	*	as AM906005	-	
Lepraria neglecta (Nyl.) Lettau		Czech Republic	Peksa 933		as AM906005	-	
Lepraria neglecta (Nyl.) Lettau		Czech Republic	Peksa 857		as AM906005	-	
clade A9							**clade 19**
Lepraria alpina (de Lesd.) Tretiach & Baruffo		Spain	Peksa 860		FN556035	FN556048	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 529	*	FN556036	-	
clade A10							**clade 7**
Lepraria incana (L.) Ach.		Czech Republic	Peksa 886		FN556037	-	
Lepraria membranacea (Dicks.) Vain.		Czech Republic	Peksa 904		FN556038	-	
Lepraria membranacea (Dicks.) Vain.		Czech Republic	Peksa 905		as FN556038	-	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 236	*	AM905997	AM906023	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 877		FM955668	FM955672	
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 851		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 852		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 853		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 854		as FM955668		

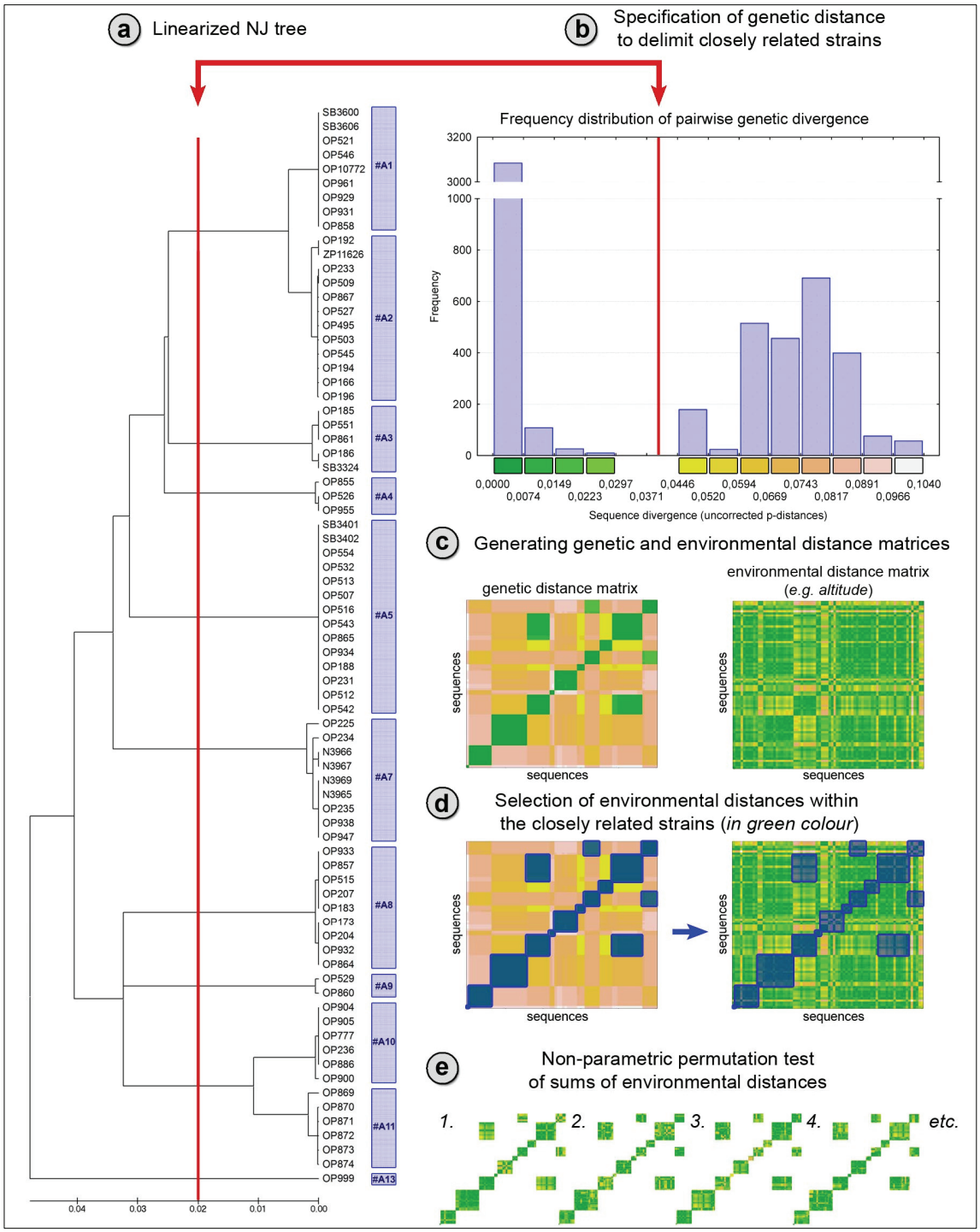
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 856		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 875		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 878		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 880		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 881		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 883		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 885		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 887		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 889		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 901		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 902		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 937		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 940		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 941		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Germany	Peksa 943		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Germany	Peksa 944		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 945		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Slovakia	Peksa 946		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Slovakia	Peksa 948		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 956		as FM955668		
Lepraria rigidula (de Lesd.) Tønsberg		Czech Republic	Peksa 900		FM955669	FM955673	
clade A11							**clade 20**
Lepraria borealis Lohtander & Tønsberg		USA, California	Peksa 869		FN556039	FN556049	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	USA, California	Peksa 868		FN556040	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	USA, California	Peksa 872		FN556041	FN556050	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	USA, California	Peksa 874		as FN556041	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	USA, California	Peksa 873		FN556042	FN556051	
Lepraria sp.		USA, California	Peksa 870		FN556043	FN556052	
Lepraria sp.		USA, California	Peksa 871		as FN556043	-	
clade A12							**clade 1 (*A. glomerata*)**
Stereocaulon sp.		Slovakia	Peksa 801		FM945392	-	
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 896		AF345404	-	
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 897		AF345405	-	
Stereocaulon pileatum Ach.		USA, Massachusetts	UTEX 1713		AF345407	-	
Stereocaulon evolutoides (H. Magn.) Frey		USA, Massachusetts	UTEX 895		AF345382	AM906024	

clade A13							**clade 2 (*A. irregularis*)**
Stereocaulon pileatum Ach.		Czech Republic	Peksa 999	*	AM905999	AM906028	
Stereocaulon sp.		Iceland	UTEX 2236		AF345411	AM906027	
Stereocaulon botryosum Ach.		USA, Alaska	Talbot 153		DQ229880	DQ229889	
Stereocaulon subcoralloides (Nyl.) Nyl.		USA, Alaska	Talbot 167		DQ229881	DQ229890	
Stereocaulon vesuvianum Pers.		USA, Alaska	Talbot 281		DQ229885	DQ229888	
others							**others**
Lepraria borealis Lohtander & Tønsberg		Czech Republic	Peksa 866		FN556044	-	
Lepraria caesioalba (de Lesd.) J.R.Laundon	chem. 1	Czech Republic	Peksa 544	*	FN556045	-	
Lepraria sp.		Slovakia	Peksa 518	*	FN556046	-	
Stereocaulon tomentosum Fr.		Slovakia	Peksa 786		FM945391	-	
Lepraria nylanderiana Kümmerl. & Leuckert		Italy, Sardinia	Nöske 246		EU008683	-	
Stereocaulon dactylophyllum Flörke		USA, Vermont	UTEX 1714		AM905993	AM906019	clade 5 (*A. excentrica*)
Stereocaulon dactylophyllum Flörke		Canada, NS	Normore 375		AF345442	-	
Stereocaulon paschale (L.) Hoffm.		USA, Alaska	Talbot 101		DQ229887	DQ229891	clade 12
Stereocaulon sp.		Costa Rica, San José	Nelsen 2181b		DQ229884	DQ229896	clade 9
Stereocaulon tomentosum Fr.		USA, Alaska	Talbot 400		DQ229882	DQ229893	

Supporting Information Table S2. Length, variability, base composition, selected substitution models, and model parameters of different data sets.

	ITS			actin		
	ITS1	ITS2	5.8 rRNA	intron 1	intron 2	exon
Alignment length	152	196	166	227	307	125
Variable sites/parsimony informative sites (in	40/26 (26.3/17.1)	32/21 (16.3/10.7)	6/2 (3.6/1.2)	161/122 (70.9/53.7)	212/162 (69.1/52.8)	19/13 (15.2/10.4)
A	16.8	18.2	27.7	14.1	19.1	26.8
C	33.6	27.8	28.3	29.5	28.4	22.1
G	28.1	26.9	23.5	28.7	31.8	32.2
T	21.4	27.1	20.5	27.7	20.7	18.9
Model estimated[a]	HKY+I	HKY+I	F81	HKY+Γ	GTR+Γ	K80+I
I, Γ values[b]	0.5241/-	0.7741/-	0/-	0/1.6922	0/2.7044	5.4595/-

[a]Estimated by the the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b.
[b]Proportion of invariable sites (I) and gamma distribution shape parameter (Γ) as estimated by PAUP/MrModeltest 1.0b.



Supporting Information Fig. S1. Scheme of the test of phylogenetic structure using the specified genetic distance delimiting closely related strains. **a.** Linearized NJ tree inferred on 76 studied strains with known ecological data, ITS data and actin data (not all 76 samples were sequenced for actin, we supposed that the samples with totally identical ITS have identical actin sequences as well). The genetic distance is shown by the scale bar below the tree. **b.** Histogram of the frequency distribution of the pairwise genetic distances among all strains. The red lines in Fig. a and b represent the selected genetic distance (calculated using Kimura 2 parameter substitution model) delimiting the closely related strains (0.04 in both figures). **c.** Matrices of pairwise genetic distances and distances of the selected environmental variable (both for 76 studied strains used in phylogenetic analysis). The distances are given in colour gradient showed under the histogram in Fig. b. **d.** The set of photobiont pairs whose genetic distances are lower than the selected value delimiting closely related strains is given in blue. The selection is applied to the matrix of the environmental variable **e.** The existence of phylogenetic signal is tested by non-parametric permutation test. The sums of Euclidean distances of environmental data are calculated for each permutation, and compared with the real data (blue regions in Fig. d).

Supporting Information Appendix S1. The R script for testing the similarity of environmental data in genetically closely related species pairs.

```
#############################################################################
#              Analysis of ecological pattern in phylogenetic tree          #
#############################################################################
#                                                                           #
# Peksa, O. & Skaloud, P. (submitted).                                      #

# Script to test for possible similarity of environmental data in genetically    #
# closely related species pairs. The similarity is evaluated as the sum of       #
# distances of environmental data. The presence of ecological pattern is         #
# tested by the permutation test.                                                #
# For graphic illustration of the procedure, see Fig. S1 (Supporting information)#
#                                                                                #
# Requires two distance matrices                                                 #
# Requires uploading of the "ecodist" package                                    #
#                                                                                #
# Script is written for R (www.r-project.org) by Pavel Skaloud, January, 2011    #
# Department of Botany, Charles University in Prague, Benatska 2,                #
# 12801 Praha 2, Czech Republic, email: skaloud@natur.cuni.cz                    #
#                                                                                #
##################################################################################
#                          Parameters to be specified                            #
##################################################################################
# nperm             = number of permutations                                     #
# nrow              = number of rows in matrices                                 #
# matrix1           = name of a full matrix of genetic distances                 #
# matrix2           = name of a full matrix of environmental distances           #
# selected.distance = selected genetic distance delimiting the closely related   #
#                     strains                                                    #
##################################################################################

library(ecodist)

#specification of required parameters
nperm             =VALUE   #e.g. 10000
nrow              =VALUE   #e.g. 76
matrix1           =NAME    #e.g. "matrix1.txt"
matrix2           =NAME    #e.g. "matrix2.txt"
selected.distance =VALUE   #e.g. 400

#import of genetic and environmental distance matrices
m1 <- matrix(scan(matrix1, n = nrow*nrow), nrow, nrow, byrow = TRUE)
   g.distance = lower(m1)
m2 <- matrix(scan(matrix2, n = nrow*nrow), nrow, nrow, byrow = TRUE)
   m.distance = lower(m2)

#counting sum of environmental distances within lineages
tested.distances <- which (g.distance<selected.distance)
morpho.dist <- m.distance [tested.distances]
suma <- sum(morpho.dist)

#permutation test
results <- vector(mode="numeric", length=nperm)
  for (xx in 1:nperm)
    {
    results [xx] <- sum(sample(m.distance, size=length(tested.distances), replace=FALSE))
    }

# counting p-value
p.results <- c(suma, results)
p.results.sort <- sort(p.results)
p.rank <- which(p.results.sort==suma)
p.value <- p.rank/nperm
p.value
```

Paper 4

Škaloud P. & **Peksa O.** (2008): Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta). – *Biologia* 63/6: 873–880.
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Comparative study of chloroplast morphology and ontogeny in *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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Abstract

Confocal laser scanning microscopy was utilized to compare the chloroplast morphology and ontogeny among five strains of the green alga *Asterochloris*. Parsimony analysis inferred from the rDNA ITS sequences confirmed their placement in three distinct lineages: *Asterochloris phycobiontica*, *Trebouxia pyriformis* and *Asterochloris* sp. Examination by confocal microscopy revealed the existence of interspecific differences in the chloroplast ontogeny of *Asterochloris*; this was based upon either specific chloroplast structures observed in a single species, or on the differential timing of particular ontogenetic sequences. The occurrence of flat parietal chloroplasts prior to cell division, considered as a basic morphological discriminative character of *Asterochloris*, was clearly associated with the process of aplanosporogenesis. By contrast, chloroplast transformation prior to the formation of autospores proceeded simply by the multiple fission of the chloroplast matrix in the cell lumen.

Key words: *Asterochloris*; *Trebouxia*; chloroplast morphology; confocal microscopy; ITS; molecular phylogeny

1. Introduction

The Swiss botanist Schwendener (1867) was the first to demonstrate that the microscopic green bodies in lichen thalli, the so-called gonidia, are in fact green or blue-green algae. Prior to that, lichenologists thought that the green bodies originated from the tips of colorless hyphae, even though their resemblance to algae was noticed. At present, an estimated 100 species in 40 genera of algae are reported as photobionts of various lichen species (Tschermak-Woess 1988; Friedl & Büdel 1996). In the majority of the associations, the phycobiont belongs to one of three genera, namely: *Trebouxia* Puymaly sensu lato, *Trentepohlia* Martius and *Nostoc* Vaucher ex Bornet et Flahault.

Among the leading researchers in lichen symbiosis was Elisabeth Tschermak-Woess (1917–2001) who greatly increased our knowledge of the morphology and systematics of

many photobionts. Her extensive scientific work includes descriptions and morphological observations of some novel or rare photobiont species, e.g. those of the genera *Dictyochloropsis* Geitler, *Myrmecia* Printz, *Trebouxia* Puymaly and *Elliptochloris* Tschermak-Woess. In particular, she was recognized as an exceptional cytologist, sometimes working at the limits of the laws of optics (for more information see Hesse 2001). In 1980, she described a new algal genus and species, *Asterochloris phycobiontica* Tschermak-Woess, based on her observations of the phycobiont of lichen *Anzina carneonivea* (Anzi) Scheidegger (Tschermak-Woess 1980). She delimited the genus as having a mainly parietal, radially lobed cup-shaped chloroplast ("sternförmig gegliederten Bechers") with a single large, or up to seven additional pyrenoids. Later however, she recognized the close relationship of *A. phycobiontica* with those species of *Trebouxia* that reproduce only by means of aplanospores. In accordance with these observations, she transferred *A. phycobiontica* into the genus *Trebouxia* subg. *Eleutherococcus* (Warén) Tschermak-Woess under the designation *Trebouxia phycobiontica* (Tschermak-Woess) Tschermak-Woess (Tschermak-Woess 1989). Additionally, Tschermak-Woess did not except the possible future elevation of the subgenera *Trebouxia* and *Eleutherococcus* as two separate genera; in that case, she suggested using the generic name *Asterochloris* for those species producing no autospores (Tschermak-Woess 1989).

Soon afterwards, ensuing molecular investigations revealed the polyphyly of the genus *Trebouxia* (DePriest 2004). Initially, Friedl & Zeltner (1994), Friedl (1995) and Friedl & Rokitta (1997) inferred from nrSSU and nrLSU rDNA sequence data that *Trebouxia magna* Archibald was more closely related to *Myrmecia biatorellae* Tschermak-Woess & Plessl than to *Trebouxia* s. str. In the light of this fact, Friedl (unpubl.) proposed a split of the genus *Trebouxia* into two genera, *Asterochloris* and *Trebouxia*, on the basis of congruencies found between morphology and DNA sequence analyses. In parallel, Rambold et al. (1998) referred to the lichen selectivity towards these two genera, assuming that all *Asterochloris* species would be the only compatible photobionts for the majority of the Cladoniaceae. Validity of *Asterochloris* was later supported by Piercey-Normore & DePriest (2001), who compared the nuclear internal transcribed spacer (ITS) sequences of many lichen photobionts and algal cultures. They revealed pairwise ITS sequence similarities among the *Asterochloris* taxa greater than 93%. Moreover, these sequences could not be aligned with those of *Trebouxia* s.

str. Therefore, it appears that the *Asterochloris* algal symbionts are distinct from those of *Trebouxia* s. str. as proposed by Friedl (unpubl.).

Eight species are presently considered to be affiliated with the genus *Asterochloris*, based on ITS sequences and morphological characteristics (Piercey-Normore & DePriest 2001; Friedl & Gärtner 1988), including *Asterochloris phycobiontica* Tschermak-Woess, *Trebouxia erici* Ahmadjian, *T. excentrica* Archibald, *T. glomerata* (Warén) Ahmadjian, *T. italiana* Archibald, *T. irregularis* Hildreth et Ahmadjian, *T. magna* and *T. pyriformis* Archibald. In addition to considerably different ITS sequences, *Asterochloris* species can be recognized by their distinctive chloroplast ontogeny, as compared to *Trebouxia*. The chloroplasts of *Asterochloris* may flatten and assume a parietal position prior to cell division, while chloroplasts of *Trebouxia* species remain lobed and at a more central position during division (Ahmadjian 1960; Hildreth & Ahmadjian 1981; Friedl & Gärtner 1988).

In the present study, we observed the chloroplast morphology and ontogeny of selected *Asterochloris* species, using both type cultures and our own isolates from the lichen *Lepraria* Acharius. The main goals of this study were to investigate the process and function of chloroplast flattening prior to cell division, and to describe some additional patterns in chloroplast morphology that are typical for genus *Asterochloris*. A combination of conventional light microscopy and confocal microscopy was utilized to better observe the morphological variations of chloroplasts during cell ontogeny in detail.

2. Material and methods

2.1. Species sampling and algal cultures

Thallus fragments of three lichenized fungi, *Lepraria borealis*, *Lepraria neglecta* and *Lepraria* sp., were collected at various localities in Central Europe (Table 1). The algal symbionts were isolated into axenic culture according to the thallus fragmentation method of Ahmadjian (1993). Cultured strains of the isolated photobionts are maintained in the private culture collection of O. Peksa at the Department of Botany, Charles University in Prague. In addition, the type strains of *Asterochloris phycobiontica* and *Trebouxia pyriformis* were obtained from the Culture Collection of Algae at the University of Göttingen (SAG) and the Culture Collection of Algae at the University of Texas at Austin (UTEX), respectively (Table 1). Observations of the algal isolates were made on cultures grown on 2% agar slants of Bold´s Basal Medium (BBM) as modified by Bischoff & Bold (1963). All cultures were

grown under standard conditions: at a temperature of 15 °C, under an illumination of 5-15 μmol m^{-2} s^{-1} in a Helkama C5G cool box.

2.2. Light and confocal microscopy

Observations using a conventional light microscope and a confocal microscope were made regularly at 7 day intervals on 2–11 week old cultures. The pure algal samples were examined by a Leica TCS SP2 confocal laser scanning microscope, equipped with an Argon-Krypton laser, using a 488 nm excitation line and an AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion objective fitted on a Leica DM IRE2 inverted microscope was used. A series of optical sections through chloroplasts were captured and used for 3-dimensional reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for the visualization of the chloroplast structure. For the final image processing we used Leica Confocal Software, version 2.61 (Leica Microsystems Heidelberg GmbH) and the Image J 1.34p program (Abramoff et al. 2004).

2.3. DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from lyophilized algal cultures following the standard CTAB protocol (Doyle & Doyle 1987), with minor modifications. Algal DNA was resuspended in sterile dH2O and amplified by the polymerase chain reaction (PCR). The ITS1, ITS2, and 5.8S rDNA regions were amplified using the algal-specific primer nr-SSU-1780-5′ (5′-CTG CGG AAG GAT CAT TGA TTC-3′; Piercey-Normore & DePriest 2001) and a universal primer ITS4-3′ (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al. 1990). All PCR were performed in 20 μl reaction volumes (15.1 μl sterile Milli-Q Water, 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 $MgCl_2$, 1 μl of DNA (5 ng/ml)). After an initial denaturing step at 95 °C for 5 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min were performed, followed by a final extension at 72 °C for 7 min. The PCR products were quantified on 1% agarose gel stained with ethidium bromide and cleaned with GENOMED Jetquick kit. The purified amplification products were sequenced with a set of sequencing primers described above (nr-SSU-1780-5′ and ITS4-3′) using the protocol for the DNA sequencing kit (ABI Prism Big-

Dye terminator cycle sequencing ready reaction, Applied BioSystems). Purified sequencing reactions were run on 3100-Avant Genetic Analyzer (Applied BioSystems). Sequencing reads were assembled and edited using SeqAssem (SequentiX Software). Newly obtained sequences were deposited in the EMBL Nucleotide Sequence Database with following accession numbers: AM900490 (*Asterochloris phycobiontica*, SAG 26.81), AM900491 (*Asterochloris phycobiontica*, LEP 9), AM900492 (*Asterochloris* sp., LEP 10), AM900493 (*Asterochloris* sp., LEP 36).

2.4. Sequence alignment and phylogenetic analyses

After initial automatic alignment using ClustalX 1.83 (Thompson et al. 1997), the ITS rDNA sequences were manually aligned using MEGA 3.1 (Kumar et al. 2004) with the following reference sequences taken from GenBank: AF345382 (*Trebouxia glomerata* UTEX 895), AF345404 (*Trebouxia glomerata* UTEX 896), AF345405 (*Trebouxia glomerata* UTEX 897), AF345406 (*Trebouxia pyriformis* UTEX 1712), AF345407 (*Trebouxia pyriformis* UTEX 1713), AF345411 (*Trebouxia irregularis* UTEX 2236), AF345423 (*Trebouxia magna* UTEX 67), AF345433 (*Trebouxia excentrica* UTEX 1714), AF345439 (*Trebouxia erici* UTEX 910), AF345440 (*Trebouxia erici* UTEX 911), AF345441 (*Trebouxia erici* UTEX 912). Positions with deletions in most sequences were removed from the alignment, resulting in an alignment comprising 533 base positions. Alignment is available from EMBL-EBI (Accession No. ALIGN_001226). The phylogenetic tree was inferred from the aligned sequence data by the maximum parsimony (MP) method using the PAUP* 4.0b10 (Swofford 2003). Reliability of the resulting topology was tested using bootstrap analysis (10 000 replications). MP phylogenies were constructed using the branch-and-bound search option, with the simple addition of sequences and gap characters treated as a fifth base.

3. Results

The morphology of the isolated photobionts was compared with that of the type strains of *Asterochloris phycobiontica* and *Trebouxia pyriformis*. Comparisons under light microscopy revealed many shared morphological features, such as: the pyriform cell shape, chloroplast flattening prior to cell division, and frequent aplanosporogenesis. Further, the high similarity of photobiont ITS sequences with all available sequences from cultured strains of *Asterochloris* corroborated the assignment of the studied *Lepraria* photobionts to the genus

Asterochloris, and revealed the close relationship among all studied strains. Parsimony analysis of the ITS data set recovered 14 most-parsimonious trees with a length of 33 steps. The resulting unrooted phylogeny of one of the most parsimonious trees is shown in Fig. 1. The tree topology corresponded with the results of Piercey-Normore & DePriest (2001), distinguishing the species *Trebouxia glomerata*, *T. pyriformis* and *T. irregularis* (Clade I sensu Piercey-Normore & DePriest) from all other species (bootstrap support 99%). The sequence of strain LEP 9 was identical with the type species of *Asterochloris phycobiontica* SAG 26.81. The ITS sequences of LEP 10 and LEP 36 were identical, thus, indicating that they formed a distinct branch separate from lineages representing other species (bootstrap value 99%). To investigate chloroplast ontogeny in *Asterochloris*, algal strains from three different evolutionary lineages were chosen: *A. phycobiontica* (strains SAG 26.81 and LEP 9), *T. pyriformis* (strain UTEX 1712) and *Asterochloris* sp. (strains LEP 10 and LEP 36).

Asterochloris phycobiontica (SAG 26.81, LEP 9)

Young cells had a central crenulate chloroplast with many simple lobes and a central pyrenoid (Fig. 2). During cell growth, the chloroplasts either retained a crenulate form with a central mass of chloroplast matrix (Figs 3, 4), or had several deep incisions that cut the outer chloroplast layer into several separate lobes (Fig. 5). Very early in the cell ontogeny, the central asteroid chloroplast assumed a parietal position (Fig. 6). However, despite the eccentric chloroplast position, the simple crenulate chloroplast lobes were evenly distributed under the cell wall (Fig. 7). In the fully parietal stage, the chloroplast margin extended into simple, finger-like lobes, that were frequently divided (Fig. 8). Simultaneous to the formation of these lobes, the chloroplast surface simplified, as the superficial lobes decreased in size. Finally, the chloroplast assumed a parietal position, with the margins extended into the finger-like lobes (Fig. 9).

In conjunction with the above-mentioned processes, the chloroplast structure underwent distinct changes prior to aplanosporogenesis. Initially, the single pyrenoid divided equally (Fig. 10) giving rise to 2–4 pyrenoids within the chloroplast. These pyrenoids assumed opposite positions in the cell and became the centres of the new daughter chloroplasts. The chloroplast matrix usually occupied the area around the pyrenoids leading to the division of the chloroplast into several parts. The new chloroplasts had a smooth surface and simple undulated margins (Figs 11, 12). Further chloroplast multiplication was signalled by further

pyrenoid divisions, and by increased complexity of the chloroplast surface. The chloroplasts migrated towards the cell centre and their surface was divided into the characteristic elongated lobes (Fig. 13). Finally, at the end of aplanosporogenesis, the chloroplast was separated into more than one hundred simple parts, entirely filling the cell lumen (Fig. 14).

Trebouxia pyriformis (UTEX 1712)

In young cells, the chloroplast assumed a central position with several lobes radiating towards the cell's periphery. The lobes were clearly extended longitudinally at their ends, leading to an elongate appearance in surface view. Terminal expansion was characterized by a T-shaped profile of the lobes as viewed in confocal optical sections (Fig. 15). Mature cells exhibited central chloroplasts with an asteroid or crenulate shape. Asteroid chloroplasts were characterized by deep lobes, emerging directly from the thin chloroplast layer spreading around the pyrenoid (Fig. 16). During cell growth, the chloroplast lobes branched both inside the cell and at the cell periphery (Fig. 17). The lobes then started to appear flattened over their entire length, with flat terminal portions of variable shape (Fig. 18). Concurrently with the above-mentioned increase of chloroplast complexity, the pyrenoids multiplied within the chloroplast matrix (Fig. 19). Before aplanosporogenesis, the chloroplast assumed a parietal position and began to divide (Fig. 20). The resulting smooth chloroplasts assumed an extremely flat shape, with no pyrenoids observed inside (Fig. 21). Finally, further chloroplast multiplication led to the formation of many simple chloroplast parts, entirely filling the cell lumen (Fig. 22).

Asterochloris sp. (LEP 10, LEP 36)

As in *T. pyriformis*, the chloroplasts of young cells assumed a central position with several lobes spreading to the cell periphery. Elongate ends of the lobes were characterized by a T-shaped profile, as viewed in confocal optical sections (Fig. 23). Rarely, a centrally positioned crenulate chloroplast with many simple lobes was present (Fig. 24). Larger cells displayed a typical asteroid chloroplast with deep lobes that emerged directly from the thin chloroplast layer spreading around the pyrenoid (Figs 25, 26). Mature cell chloroplasts exhibited several ontogenetic stages, alternating during the cell's ontogeny. Ordinary chloroplast lobes could change into the flattened ones, with flat peripheral endings of variable shapes (Fig. 27). Alternatively, the chloroplast's surface was sometimes cut into many tubular branched lobes

(Fig. 28). Finally, some cells were characterized by having a sun-like shaped chloroplast, formed by many thin radial lobes that emerged from the deep chloroplast layer (Fig. 29).

All the above-mentioned chloroplast stages could change as a consequence of the processes associated with asexual reproduction. Initial stages of aplanosporogenesis were signalled by multiplication of the pyrenoid, followed by the displacement of the chloroplast to a parietal position (Fig. 30). Then, the chloroplast matrix occupied the area around the pyrenoids, leading to the division of the chloroplast. The resulting smooth surfaced chloroplasts further broke up into a large number of simple parts, filling up the cell lumen (Fig. 31). By contrast, autospore production was characterized by the direct fission of a central asteroid chloroplast into several parts, without any migration to a parietal position (Figs 32, 33). During the subsequent separation the resulting chloroplast parts filled up the whole cell lumen.

4. Discussion

Molecular analysis of the ITS rDNA sequences clearly placed all investigated photobionts in three distinct lineages within *Asterochloris*. Strains LEP10 and LEP 36 formed a lineage that was separate from all described species as well as from published *Asterochloris* sequences. Therefore, they very probably represent a new species of *Asterochloris* (Fig. 1). *Trebouxia italiana*, the last described species of *Asterochloris*, with no published sequence, has a very different chloroplast morphology and cell dimensions compared to both investigated strains (Gärtner 1985). These results indicate the presence of obvious cryptic species diversity in *Asterochloris*, as has been recently shown in other trebouxiophycean clades (Kroken & Taylor 2000; Neustupa et al. 2007).

Although it is undeniable that molecular characteristics play a leading role in the taxonomy of *Trebouxia* s.l., chloroplast morphology is still regarded as an important criterion in species delimitation (Beck et al. 1998; Friedl & Rokitta 1997). Despite the existance of different taxonomic concepts in *Trebouxia* s.l., the heterogeneity of *Trebouxia* was often demonstrated by the conspicuous differences in chloroplast structure. One of the main disparities, also applied recently for *Trebouxia* and *Asterochloris* separation, was the occurrence of flat parietal chloroplasts prior to cell division (Hildreth & Ahmadjian 1981; Friedl & Gärtner 1988). Our observations confirm the validity of this distinction, as the ontogenetic stage with flat parietal chloroplasts was noticed in all studied strains of *Asterochloris* (Figs 12, 21, 30).

Moreover, further examination of mature cells revealed the specific occurrence of parietal chloroplasts only in the initial stages of aplano- or zoosporogenesis. On the other hand, chloroplast transformation prior to the formation of autospores occurred without chloroplast flattening, and simply involved the multiple fission of the chloroplast matrix in the cell lumen (Figs 32, 33). These observations appear to suggest the existence of two distinct ontogenetic pathways leading to chloroplast splitting prior to cell division. In the case of aplano- and zoosporogenesis, a large number (up to 128) of daughter cells is created compared to the production of autospores (Fig. 31). The requirement for a chloroplast to split into a large number of equal parts can lead to the necessity of chloroplast simplification prior to this process. The importance of this simplification can be demonstrated by *A. phycobiontica*: although the divided chloroplasts have a complicated structure with a lobed surface (Figs 13, 14), the splitting of the chloroplast precedes the formation of flat parietal chloroplasts with undulate margins (Figs 11, 12). Interestingly, these parietal chloroplasts have also been observed in *T. erici*, *T. glomerata*, *T. irregularis* and *T. pyriformis* (Friedl & Gärtner 1988), thus, in the majority of *Asterochloris* species. However, the specific stage of cell ontogeny demonstrating parietal chloroplasts has never been observed in *Trebouxia*, despite the evident prevalence of zoospores in this genus (Gärtner 1985). It would be interesting to compare the chloroplast ontogeny in *Asterochloris* and *Trebouxia* in greater detail which could clarify whether the parietal stage occurs in *Trebouxia,* or whether morphological transformation of chloroplasts during the process of aplano- and zoosporogenesis proceeds via a different ontogenetic pathway.

In addition to the above-mentioned stage characterized by flat parietal chloroplasts, there are some further morphologically identical stages between either all three species studied or at least two of them. These include a central axial chloroplast with elongate lobes that are T-shaped in profile (Figs 15, 23), a massive crenulate chloroplast with many simple lobes (Figs 3, 4, 24), a central chloroplast with deep long lobes (Figs 5, 16, 25, 26), and an axial chloroplast with flattened lobes terminated by flat peripheral endings of variable shape (Figs 18, 27). Although these stages are shared amongst the species of *Asterochloris*, specific differences primarily concern the different timing of the particular stages in chloroplast ontogeny. However, since we did not study synchronized cultures, we were not able to precisely time the occurrence and duration of particular stage within cell ontogeny. The results presented here correspond with the observations of Škaloud et al. (2005), who

identified several distinct ontogenetic stages shared by species of coccal green alga *Dictyochloropsis* Geitler. However, we found that certain specific chloroplast developmental stages occurred in one species only, for example, the simple lobed chloroplast margin of mature cells and the parietal position of chloroplasts in *A. phycobiontica*.

Although the results of molecular investigations demonstrated the polyphyly of *Trebouxia* and clearly segregated the genus *Asterochloris* (Piercey-Normore & DePriest 2001; DePriest 2004), the morphological diagnostic criteria of individual species remain vague. We hope that the distinctive differences in chloroplast ontogeny as demonstrated in this study will form a useful contribution towards future combined structural/molecular taxonomic investigations that are aimed at developing a clear species and genus concept in *Trebouxia* and *Asterochloris*.

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Table 1. Species and strains of *Asterochloris* used in this study.

Phycobiont	Strain no. [a]	Isolated from lichen	Locality	Collector	Year
Asterochloris phycobiontica	SAG 26.81	*Anzina carneonivea*	Italy, Trento, Madonna di Campiglio.	Tschermak-Woess E.	1976
Asterochloris phycobiontica	LEP 9	*Lepraria neglecta*	Ukraine, East Carpathians, Breskul Mt.	Slavíková Š.	2004
Asterochloris sp.	LEP 10	*Lepraria borealis*	Bulgaria, Stara planina Mts, Central Balkan NP.	Slavíková Š. & Slavík M.	2004
Asterochloris sp.	LEP 36	*Lepraria* sp.	Czech Republic, Máslovická stráň NR.	Peksa O. & Jindráková Z.	2006
Trebouxia pyriformis	UTEX 1712	*Cladonia squamosa*	USA, Massachusetts, Leverett	Hutchinson W.A.	1969

[a] SAG - culture collection of algae at the University of Göttingen (http://www.epsag.uni-goettingen.de/html/sag.html); UTEX - culture collection at the University of Austin, Texas (http://www.bio.utexas.edu/research/utex/); LEP – authors' strain designation.

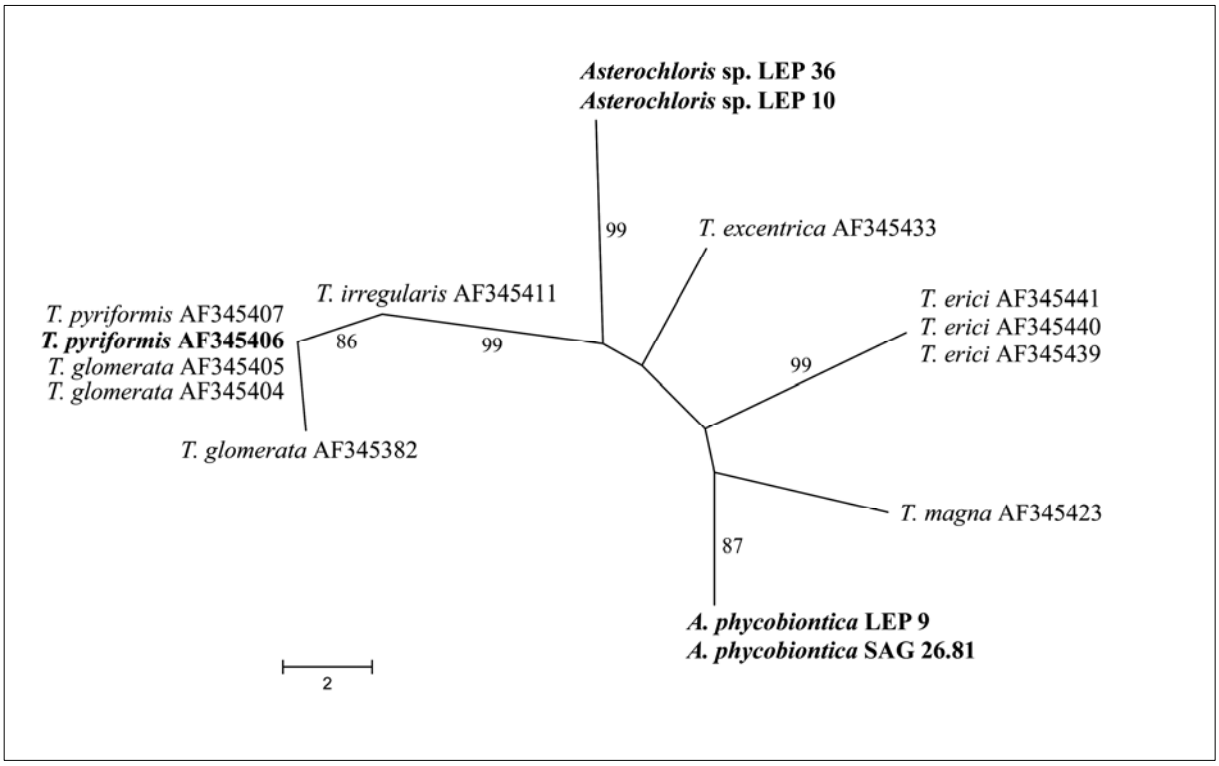
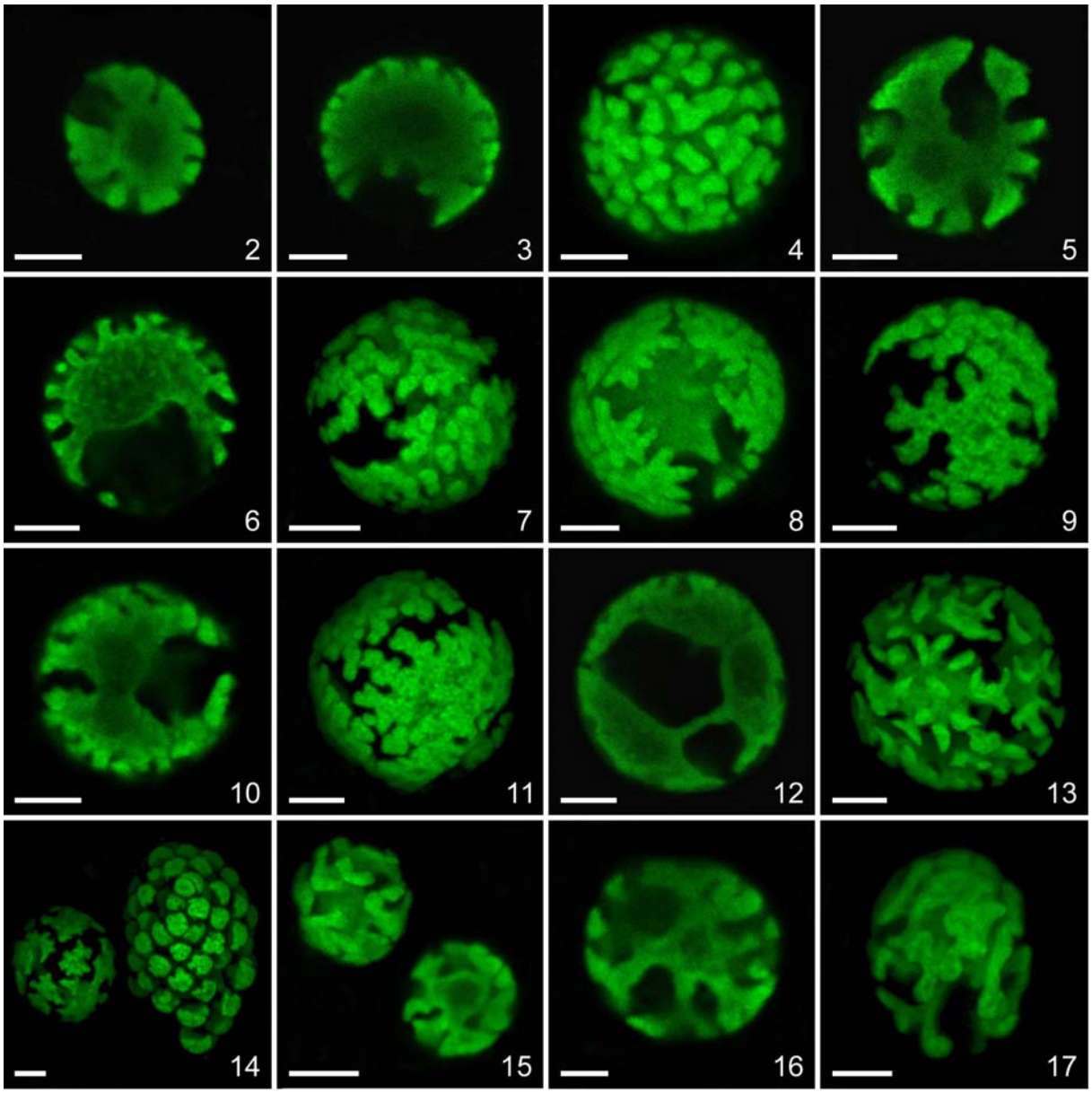
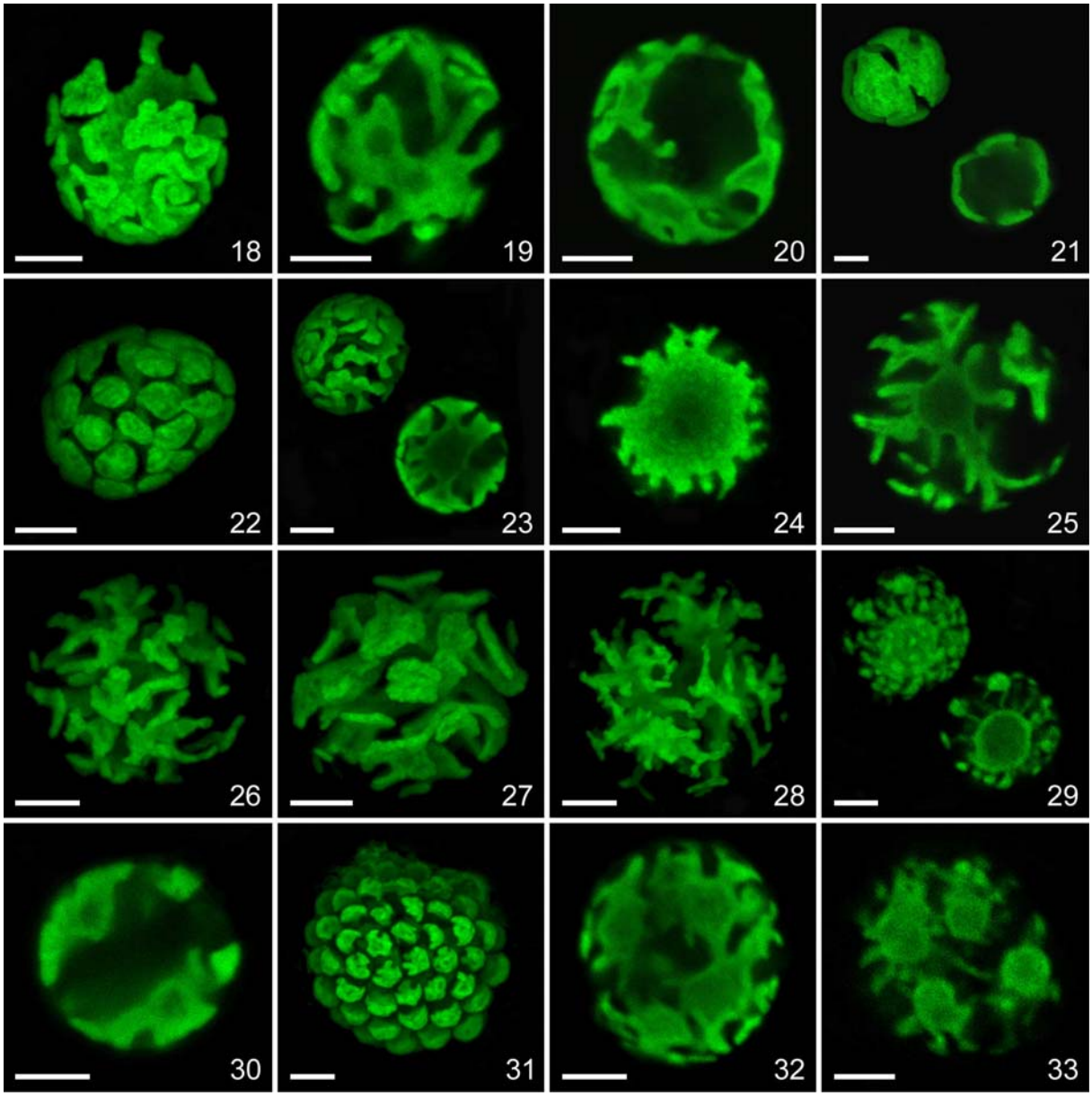


Fig. 1. Unrooted phylogeny of *Asterochloris* ITS rDNA sequences using the maximum parsimony method and a branch-and-bound search. Values at the nodes represent statistical support estimated by maximum parsimony bootstrapping. The scale bar indicates the distance due to two evolutionary steps. Investigated strains are indicated in **bold**.



Figs 2–17. Confocal sections (CS) and maximum projections (MP) of chloroplast. 2-14: *Asterochloris phycobiontica*. 2 – simple chloroplast of young cell (CS); 3 – crenulate chloroplast (CS); 4 – crenulate chloroplast (MP); 5 – axial chloroplast with deep lobes (CS); 6 – parietal position of chloroplast (CS); 7 – chloroplast surface with many simple lobes (MP); 8 – parietal chloroplast with finger-like lobes (MP); 9 – smooth chloroplast surface with divided marginal lobes (MP); 10 – pyrenoid multiplication (CS); 11 – dividing of smooth parietal chloroplast (MP); 12 – chloroplast division into two parts (CS); 13 – lobed surface of divided chloroplast parts (MP); 14 – aplanospore production (MP). 15–17: *Trebouxia pyriformis*. 15 – simple chloroplast of young cells (MP, CS); 16 – deeply lobed axial chloroplast (CS); 17 – chloroplast with branched lobes (MP). Scale bar: 5 μm.



Figs 18–33. Confocal sections (CS) and maximum projections (MP) of chloroplast. 18–22: *Trebouxia pyriformis*. 18 – flattened chloroplast lobes with flat terminal parts (MP); 19 – pyrenoid multiplication (CS); 20 – parietal position of chloroplast (CS); 21 – smooth parietal chloroplast during its division (MP, CS); 22 – aplanospore production (MP). 23–33: *Asterochloris* sp. 23 – simple chloroplast of young cells (MP, CS); 24 – crenulate chloroplast (CS); 25 – deeply lobed chloroplast (CS); 26 – deeply lobed chloroplast (MP); 27 – flattened chloroplast lobes with flat terminal parts (MP); 28 – chloroplast surface consisted of tubular lobes (MP); 29 – sun-like chloroplast (MP, CS); 30 – flattened parietal chloroplasts (CS); 31 – aplanospore production (MP); 32, 33 – chloroplast division during the autosporogenesis (CS). Scale bar: 5 μm.

Paper 5

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Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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Abstract

The genus *Asterochloris* is one of the most common lichen photobionts. We present a molecular investigation of 41 cultured strains, for which nuclear-encoded ITS rDNA and partial actin I sequences were determined. The loci studied revealed considerable differences in their evolutionary dynamics as well as sequence variation. As compared to ITS data, the actin sequences show much greater variation, and the phylogenies yield strong resolution and support of many internal branches. The partitioning of ITS dataset into several regions yielded better node resolution in both markers, especially in ITS dataset. We recognized 16 well-supported monophyletic lineages, of which one represents the type species of the genus (*Asterochloris phycobiontica*), and six correspond to species previously classified to the genus *Trebouxia* (*T. erici*, *T. excentrica*, *T. glomerata*, *T. irregularis*, *T. italiana* and *T. magna*). Only 15% of isolated photobionts considered in our study could be assigned with certainty to previously described species, emphasizing amazing cryptic variability in *Asterochloris*. Concurrently with the formal delimitation of the genus *Asterochloris*, we propose new combinations for the former *Trebouxia* species; furthermore, *T. pyriformis* is reduced to a synonym of *A. glomerata*. The present knowledge of global diversity of *Asterochloris* algae is discussed.

Keywords: actin; *Asterochloris*; complementary base changes (CBC); cryptic diversity; doublet model; ITS rDNA; lichen photobionts; likelihood mapping; RNA secondary structure; substitutional saturation; taxonomy, Trebouxiophyceae

1. Introduction

Lichens are prime examples of symbiotic associations composed of a fungal (mycobiont) and a photosynthetic (photobiont) partner that may be either a green alga or cyanobacterium. The Swiss botanist Schwendener (1867) was the first to demonstrate that the microscopic green bodies in lichen thalli, the so-called gonidia, were in fact green algae or cyanobacteria. Prior to that, lichenologists thought that the green bodies originated from the tips of colorless

hyphae, even though their resemblance to algae was noticed. At present, an estimated 100 species in 40 genera of algae are reported as photobionts of various lichen taxa (Friedl and Büdel, 2008; Tschermak-Woess, 1988). The most common photobiont genus, *Trebouxia*, is present in approximately 20% of all lichen species (DePriest, 2004; Tschermak-Woess, 1988).

Since the beginning of investigations of *Trebouxia* algae, some researchers found that the genus was heterogenous, and recognized the existence of two species groups. Initially, Warén (1920) established two subgenera based on differences in asexual reproduction. Those algae which produced 4–16 daughter cells tightly adjoined to the sporangial cell wall he designated as *Eucystococcus*, and those reproducing by more than 32 smaller, spherical daughter cells, he assigned to *Eleuterococcus*. Ahmadjian (1959a, 1960) divided *Trebouxia* into two main groups depending on the position of the chloroplast prior to sporogenesis as well as on cell shape. Group I was characterized by a parietal position of the chloroplast prior to cell division and rather oval cells, while group II was defined by a central position of dividing chloroplasts and rather spherical cells. Moreover, chloroplasts of group I algae were deeply lobed, with lobes reaching the cell periphery, as compared to group II algae containing a rather massive central chloroplast with a smoother surface. This differentiation was further confirmed by Jacobs and Ahmadjian (1968) and Peveling (1968) based on ultrastructural comparison of pyrenoid structure. The differences consisted of rather arcuate (group I) or swollen (group II) thylakoids penetrating the pyrenoid matrix and on a highly vesiculate pyrenoid in group II. Later, Archibald (1975) distinguished the genera *Pseudotrebouxia* and *Trebouxia*, based on the differences in cell wall characters during asexual reproduction (Groover and Bold, 1969). However, Gärtner (1985a, b) rejected establishment of *Pseudotrebouxia* because his observations did not confirm Archibald's conclusions. Moreover, recent molecular data confirm Gärtner's rejection of *Pseudotrebouxia* (e.g. Kroken and Taylor, 2000).

In 1980, Tschermak-Woess described a new genus and species, *Asterochloris phycobiontica*, based on her observations of the photobiont of a lichen *Anzina carneonivea* (Tschermak-Woess, 1980; as *Varicellaria carneonivea*). Although some morphological features were similar to *Trebouxia* species, she delimited the genus as having a mainly parietal, radially lobed, cup-shaped chloroplast with a single large, or up to seven additional, pyrenoids. Later, when revising the taxonomy of *Trebouxia*, she split the genus into two subgenera, *Trebouxia* and *Eleutherococcus* (Tschermak-Woess, 1989). In her opinion, Eleutherococcus was defined by the strict absence of autospores, which occurred in subg. Trebouxia. Concurrently, she transferred *A. phycobiontica* into the genus *Trebouxia* subg. *Eleutherococcus* under the designation *Trebouxia phycobiontica*. Additionally, Tschermak-

Woess did not exclude the possible of future reclassification of *Trebouxia* subgenera (*Trebouxia* and *Eleutherococcus*) into two separate genera; in that case, she suggested using the generic name *Asterochloris* for those species producing no autospores (Tschermak-Woess, 1989).

Soon afterwards, molecular investigations revealed that genus *Trebouxia* is paraphyletic with *Myrmecia*. Initially, Friedl and Zeltner (1994), Friedl (1995) and Friedl and Rokitta (1997) inferred from nrSSU and nrLSU sequence data that *Trebouxia magna* and *Trebouxia erici* were more closely related to *Myrmecia biatorellae* than to *Trebouxia* s. str. In light of this, a split of the genus *Trebouxia* into two genera, *Asterochloris* and *Trebouxia*, was proposed (Friedl unpublished observations; in Rambold et al., 1998; Helms et al., 2001) based on the suggestion made by Tschermak-Woess (1989). The validity of *Asterochloris* was later supported by Piercey-Normore and DePriest (2001), who compared the nuclear internal transcribed spacer (ITS) sequences of many lichen photobionts and algal cultures. They revealed pairwise ITS sequence similarities among the *Asterochloris* taxa greater than 93%. Moreover, these sequences could not be aligned with those of *Trebouxia* s. str. or any other genera, supporting monophyly of *Asterochloris*. Finally, the 18S rDNA phylogenetic tree of lichen photobionts, including the sequences of the authentic strain of *A. phycobiontica* (SAG 26.81), *"Trebouxia" magna*, and three other *Trebouxia* s.str. species, unambiguously shows that *Asterochloris* is a monophyletic genus distinct from the most-closely related genera *Myrmecia* and *Trebouxia* (Friedl and Büdel, 2008).

Considering the distinguishing features proposed by several authors in the past, *Asterochloris* could be delimited from closely related genera (i.e. *Trebouxia* s. str. and *Myrmecia*) by means of several discriminative morphological characters: parietal position of chloroplast prior to zoo- or aplanosporogenesis (Fig. 1A; Ahmadjian, 1960; Friedl and Gärtner, 1988; Hildreth and Ahmadjian, 1981; Škaloud and Peksa, 2008); deeply lobed chloroplast (Fig. 1B; Ahmadjian, 1959b, 1960); rather oviform, elliptical and pyriform cell shape (Fig. 1C; Ahmadjian, 1960; Hildreth and Ahmadjian, 1981); and high proportion or strict presence of aplanospores, i.e. a large number (64-128) of immotile small daughter cells with cell walls (Fig. 1D; Friedl, 1993; Tschermak-Woess, 1989; Warén, 1920). In conjunction with these morphological features, *Asterochloris* could be delimited by the typical ultrastructure of its pyrenoid matrix, that consist of several thin and curved thylakoid tubules with associated pyrenoglobuli (Friedl, 1989a; Jacobs and Ahmadjian, 1968; Peveling, 1968); as well as by photobiont selection toward certain groups of lichen-forming fungi,

predominantly Cladoniineae, i.e. suborder of Lecanorales, including families Cladoniaceae and Stereocaulaceae (Ahmadjian and Jacobs, 1981; Rambold et al., 1998; etc.).

Several recent studies have illustrated that a wide range of *Asterochloris* taxa occur as photobionts of various fungal species (e.g. Beiggi and Piercey-Normore, 2007; Cordeiro et al., 2005; Nelsen and Gargas, 2008; Piercey-Normore, 2004; Piercey-Normore and DePriest, 2001; Yahr et al., 2004, 2006). Despite this, formal delimitation of *Asterochloris* as well as assignment of the species to that genus is still pending. In the present study, ITS rDNA and an intron-containing portion of the actin type I gene sequences have been determined for a variety of lichen photobiont strains to investigate the genetic diversity in *Asterochloris*. Our goals were (1) to identify genetically distinct groups of strains, (2) to compare levels of phylogenetic variation and benefits of ITS and actin loci, and (3) to formally delineate the genus *Asterochloris* and establish new combinations for former *Trebouxia* species.

2. Materials and methods

2.1. Species sampling and algal cultures

Sampled strains were chosen to represent the diversity of the algae putatively belonging to the *Asterochloris* lineage. Predominantly, algal symbionts were isolated from the lichen fungi in the Cladoniineae (genera *Lepraria* Ach., *Cladonia* P. Browne, *Stereocaulon* Hoffm.; and *Diploschistes* Norman) sampled at various localities in Central and Eastern Europe. The algal symbionts were isolated into unialgal cultures according to the thallus fragmentation method of Ahmadjian (1993). In addition, eight *Asterochloris* strains (currently known as *Trebouxia* species) were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA – UTEX 67, 902, 911, 1712, 1714, 2236; the Culture Collection of Algae at the University of Göttingen, Germany – SAG 26.81; and the Culture Collection of Algae and Protozoa, Oban, United Kingdom – CCAP 519/5B. Lichen specimens were deposited in the herbarium of O. Peksa (PL – West Bohemian Museum in Pilsen) and S. Slavíková-Bayerová (PRA – Institute of Botany, Academy of Sciences of the Czech Republic). Algal strains were deposited in the Culture Collection of algae of Charles University in Prague, Czech Republic (CAUP), either in the living form or cryopreserved in a liquid nitrogen. Algal strains were cryopreserved in a 10% dimethylsulfoxide (DMSO) using 5100 Cryo 1°C Freezing Container (Mr. Frosty, Nalgene). The 74 new sequences generated in this study were deposited in EMBL Nucleotide Sequence Database. All samples and sequences used in this study, with their accession numbers, are shown in Supplementary Table 1.

2.2. Microscopic observations

Observations of the algal isolates were made from cultures grown on 2% agar slants of Bold's Basal Medium (BBM) as modified by Bischoff and Bold (1963). All cultures were maintained at a temperature of 15 °C, under an illumination of 5-15 µmol m^{-2} s^{-1} (cooling box Helkama C5G). Individual strains were regularly observed under an Olympus BX51 light microscope to reveal morphological variability.

2.3. DNA extraction, PCR and DNA sequencing

Total genomic DNA was extracted from lyophilized algal cultures following the standard CTAB protocol (Doyle and Doyle, 1987), with minor modifications. Algal DNA was resuspended in sterile dH2O and amplified by 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). Actin type I locus (1 complete exon and two introns located at codon positions 206 and 248; Weber and Kabsch, 1994) was amplified using the published algal specific primers a-nuact1-0645-5' (5'-GAC AGA GCG TGG KTA CAG-3') and a-nu-act1-0818-3' (5'-TGA ACA GCA CCT CAG GGC A-3'; Nelsen and Gargas, 2006) and newly designed primers ActinF2 Astero (5'-AGC GCG GGT ACA GCT TCA C-3') and ActinR2 Astero (5'-CAG CAC TTC AGG GCA GCG GAA-3'). All PCR were performed in 20 µl reaction volumes (15.1 µl sterile Milli-Q Water, 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 $MgCl_2$ (25mM), 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 at 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 a 1% agarose gel stained with ethidium bromide and cleaned either with the JetQuick PCR Purification Kit (Genomed) or with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocols. The purified amplification products were sequenced with the PCR primers using the protocol for the DNA sequencing kit (ABI Prism Big-Dye terminator cycle sequencing ready reaction, Applied BioSystems). Purification of

sequencing reactions was carried out using an ethanol/sodium acetate precipitation provided with the sequencing kit. Products were run on an ABI 3100 Avant automated sequencer (Applied BioSystems). Further, sequencing of several PCR products was performed with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. Sequencing reads were assembled and edited using SeqAssem programme (SequentiX Software).

2.4. Sequence alignment and DNA analyses

Sequences were initially aligned using ClustalX 1.83 (Thompson et al., 1997) and MUSCLE (Edgar, 2004). Sequences from GenBank were acquired and included only if they met two following criteria: they represent *Asterochloris* sequences; and both ITS and actin I locus sequences were available. After deleting identical sequences, the resulting alignment comprises 37 (ITS dataset), 44 (actin dataset) or 48 (concatenated dataset) sequences, respectively (Supplementary Table 1). ITS sequences (comprised ITS1, 5.8S and ITS2 regions) were aligned on the basis of their rRNA secondary structure information (see below) with MEGA 4 (Kumar et al., 2008). The alignment of actin I locus sequences was more difficult than those of the ITS region. Although several successive MUSCLE alignments considerably improved alignment quality, some ambiguous positions remained. Due to an absence of published secondary structure of *Asterochloris* or a related genera, the stability of alignment has been assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP v.1.2 alpha 4 (Löytynoja and Milinkovitch, 2001). Gap penalties were incrementally adjusted from 7 to 17 by steps of 2, and extension penalties were adjusted from 4 to 9 by steps of 1. Regions of instability were deleted by computing to a 90% consensus among the 36 different alignments, leaving an alignment of 622 positions. The robustness of an alignment was then tested by simply comparing NJ trees constructed in MEGA, from the resulting alignment, and those created by SOAP with the opening/extension penalty parameters varied either from 7/0.04 to 17/0.2 or from 14/4 to 16/9, respectively. The resulting tree topologies were consistent, with only bootstrap values slightly differing (trees not shown). The alignments are available from the first author upon request.

The amount of phylogenetic signal vs. noise was assessed by several approaches (Verbruggen and Theriot, 2008). First, we plotted the uncorrected against corrected distances determined with the respective model of sequence evolution estimated by the program MODELTEST version 3.06 (Posada and Crandall, 1998). The selected models and model

parameters are summarized in Table 1. Also, the measure of skewness (g_1-value calculated for 10,000 randomly selected trees in the program PAUP* version 4.0b10; Swofford, 2002) was compared with the empirical threshold values (Hillis and Huelsenbeck, 1992) to verify the nonrandom structuring of the data. To quantify the extent of substitution saturation in datasets, we calculated the *Iss* statistic for all data partitions with the program DAMBE (Xia and Xie, 2001). Finally, the phylogenetic signal present in the data partitions was estimated by maximum likelihood mapping (Strimmer and von Haeseler, 1997) using the TREE-PUZZLE 5.2 program (Schmidt et al., 2002).

The phylogenetic trees were inferred with Bayesian inference (BI) using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). Three datasets were used, each comprising 60 *Asterochloris* taxa: ITS (520 bp), actin (622 bp) and the concatenated set of ITS and actin sequences (1142 bp). The most appropriate substitution model was estimated for each dataset as well as each partition within the alignment using the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander 2004). In the BI analysis, two parallel MCMC runs were carried out for 3 million generations, each with one cold and three heated chains. Four different approaches were taken. Firstly, the alignment datasets were analyzed using a single, general time-reversible model with rate variation across sites and proportion of invariable sites (GTR+Γ+I) as estimated by PAUP/MrModeltest 1.0b. Secondly, the datasets were divided into six region partitions (ITS1, ITS2, 5.8 rRNA, actin intron 206, actin intron 248, actin exon), and for each partition different substitution models were selected by PAUP/MrModeltest 1.0b (Table 1). In the third set of analyses, the datasets were divided into stem-loop (ITS dataset) or codon position (actin exon) partitions. In the ITS dataset, different substitution models were selected for stem and loop partitions, as extracted from the RNA secondary structure information. 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; Schöniger and von Haeseler, 1994) was selected (Leliaert et al., 2007; Verbruggen and Theriot, 2008). In the actin dataset, the exon partition was divided into three categories representing 1st, 2nd, and 3rd codon positions (CP model; Shapiro et al., 2006). Finally, the second and the third approach were combined, dividing the datasets into eleven partitions (ITS1-loop, ITS1-stem, 5.8-loop, 5.8-stem, ITS2-loop, ITS2-stem, actin-intron 206, actin-intron 248, actin-exon CP_1, actin-exon CP_2, actin-exon CP_3). 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.

Bootstrap percentages and posterior probabilities were interpreted as weak (less than 50 %) moderate (50–94% for BI; 50–79% for ML and MP) or high (more than 94% for BI; more than 79% for ML and MP).

Bootstrap analyses were performed by maximum likelihood (ML) and weighted parsimony (wMP) criteria using GARLI, version 0.951 (Zwickl, 2006 unpublished Ph.D. dissertation; http://www.zo.utexas.edu/faculty/antisense/Garli.html) and PAUP*, version 4.0b10, respectively. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The wMP bootstrapping (1000 replications) was performed using heuristic searches with 100 random sequence addition replicates, Tree bisection reconnection swapping, number of trees limited to 10,000 for each replicate, and gaps treated as missing data. If multiple, most parsimonious trees were recovered, a single tree was selected by using Farris, 1969 successive weighting approach with PAUP's REWEIGHT command (base-weight: 1000, rescaled consistency index, mean fit). Bootstrap percentages and posterior probabilities were interpreted as weak (less than 50%) moderate (50–94% for BI; 50–79% for ML and MP) or high (more than 94% for BI; more than 79% for ML and MP).

Congruence between separately analyzed datasets was inferred by inspecting bootstrap scores above 70% resulting from separate ML and MP analyses of the ITS and actin data set (Mason-Gamer and Kellogg, 1996). Further, congruence between datasets was tested using the incongruence length difference (ILD) test (Farris et al., 1995), as implemented by the partition homogeneity test in PAUP* (heuristic search, simple addition, TBR branching swapping, 1000 replicates). In view of several recent reports critizing the results of ILD tests (Dolphin et al., 2000; Ramirez, 2006), we also explored whether resolution and support would be improved by increasing the amount of sequencing data, by direct comparison of bootstrap supports and posterior probabilities among analyses of all three datasets.

The secondary structures of ITS rDNA sequences were constructed using the mfold computer program (version 2.3; Walter et al., 1994; Zuker, 2003), with folding temperature set to 25 °C. The structures were compared with published ITS secondary structure of *Asterochloris* photobionts (Beiggi and Piercey-Normore, 2007). The determination of stem and loop regions in 5.8 rRNA was determined according to the published 5.8 rRNA secondary structure transcript (Jobes and Thien, 1997). The common secondary structures were created using RnaViz (version 2; De Rijk et al., 2003) and used to identify compensatory base changes (CBCs) and hemi-CBCs.

3. Results

3.1. Analysis of the molecular data

Specifications of the ITS, actin and concatenated datasets and evolutionary models applied are given in Table 1. Although both loci consisted of similar numbers of nucleotide pairs analyzed, they differed considerably in the amount of phylogenetic signal. The actin dataset contained significantly higher amount of both variable and parsimony informative sites than the ITS region, which led to higher average sequence divergence and better resolution of taxa relationships (Table 2). Testing the data partitions for substitution saturation (distribution of the uncorrected vs. corrected distances; Fig. 2) revealed a nearly linear correlation in both ITS rDNA and actin data indicating low saturation. Accordingly, the I_{ss} statistics did not reveal significant saturation in any of the datasets and partitions (Table 1). In addition, the difference in the amount of phylogenetic signal was well visible when comparing genetic distances of ITS rDNA and actin loci (Fig. 2A). Only minimal differences in saturation were detected among different partitions within the two loci. In ITS rDNA, the ITS1 partition was slightly less saturated than the rest of the locus (Fig. 2B). The amount of saturation in the three actin partitions was almost identical (Fig. 2C).

Comparison of the skewness of the tree length distribution (g_1 value) of random trees of all partitions with the empirical threshold values (Hillis and Huelsenbeck, 1992) showed that the length distributions were considerably left-skewed, indicating that the alignments were significantly more structured than random data and likely contained a strong phylogenetic signal (Table 1).

Results of likelihood mapping are presented in Table 1 and Fig. 2. Within the ITS rDNA dataset the strongest phylogenetic signal was detected in the ITS1 partition (56.2% of fully resolved quartets). The phylogenetic signal of the actin dataset was better than that in the ITS rDNA data. Within the actin alignment the strongest phylogenetic signal was identified for the intron 248 partition (77.8% of fully resolved quartets). The combined alignment of both loci had the highest percentage of fully resolved quartets (79%) and only 13.8% of quartets fully unresolved.

3.2. Phylogenetic analyses

Considering the distinct differences in substitution model, model parameters and amount of phylogenetic signal among the partitions in both loci (Table 1), the phylograms were constructed by Bayesian inference on partitioned datasets (Fig. 3, Supplementary Figs 1,2). Visual inspection of single-locus trees revealed that all moderately to well-supported clades

(above 70% bootstrap) were monophyletic in both ITS and actin trees. Clearly, the data sets did not shown strong support for the conflicting rival nodes. According to the partition homogeneity test (ILD test), the ITS and actin datasets were not significantly heterogeneous ($p = 0.07$), justifying a combined data approach. The analyses of the concatenated dataset led to the increasing of average node resolutions in all inference methods (Table 2), indicating improvement of the phylogenetic signal. Regarding the above-mentioned characteristics, as well as likelihood mapping results (Table 1), we inferred concatenated ITS+actin phylogeny to better reveal the relationships among taxa.

ITS phylogram (Supplementary Fig. 1) revealed one well supported lineage (PP = 1.00) comprising authentic strains of *Trebouxia glomerata* (UTEX 895), *T. pyriformis* (UTEX 1712) and *T. irregularis* (UTEX 2236). Moreover, the analysis revealed five well-supported lineages (clades 8, 9, 11, 12, and 14 in Supplementary Fig. 1) not containing any previously described *Trebouxia* species. Although the most of the terminal clades revealed high PP support, the relationship among the clades remained unresolved. The actin phylogeny (Supplementary Fig. 2) acquired much higher bootstrap support for all terminal lineages, as well as for the internal nodes. The proportion of highly supported nodes and average node resolution was significantly larger in the actin analysis as compared to the ITS results (Table 2).

The analysis of combined ITS rDNA and actin datasets (Fig. 3) showed very similar topology to the actin tree. Both analyses led to the recognition of 16 well-resolved clades (clades #1–16 in Fig. 3), incl. three lineages containing a single authentic strain of *Trebouxia* species. As compared to the ITS phylogram, the analyses significantly discriminated between the closely related clades #1–2, and #15–16. The concatenated phylogeny also points to the genetic similarity of authentic strains of *T. glomerata* and *T. pyriformis*, and *T. italiana* and *T. magna* (strain UTEX 67), respectively. Analysis of concatenated sequence data also recovered the existence of three major, well-supported clades (clades A–C in Fig. 3): (A) a lineage composed of *T. glomerata* (UTEX 895), *T. pyriformis* (UTEX 1712), *T. irregularis* (UTEX 2236), *T. magna* (UTEX 67) and *T. erici* (UTEX 911) clades; (B) a lineage consisting of *T. excentrica* (UTEX 1714) and two novel, thus far unresolved lineages of photobionts isolated from *Lepraria* species (clades #6 and #7); and (C) a large clade containing the remaining strains, including the lineage comprising the authentic strain of *A. phycobiontica* (SAG 26.81); the pair of authentic strains of *T. italiana* (CCAP 519/5B) and *T. magna* (UTEX 902); and seven novel well-resolved lineages of mainly *Lepraria* and *Cladonia* photobionts (clades #8, 9, 10, 11, 12, 14, and 16).

3.3. ITS1 and ITS2 secondary structures

A common overall organization of the ITS1 and ITS2 rDNA secondary structures could be identified in all strains (Fig. 4). The ITS1 secondary structure comprised four paired regions (helices I–IV), with helix I to be the most divergent in sequences (Fig. 4A). The ITS2 secondary structure possessed conserved motifs among green algae (Mai and Coleman, 1997), i.e. four-fingered hand (helices I–IV), a pyrimidine–pyrimidine mismatch in helix II, and a conserved sequence of UGGU on the 5' side of helix III (Fig. 4B). The ITS secondary transcripts were compared among the 16 lineages revealed in the concatenated phylogram (Fig 3) to check the occurrence of Compensatory Base Changes (CBCs; nucleotide changes at both sides of paired bases) and hemi-CBCs (change at only one side of nucleotide pair, but still preserving pairing) according to Coleman (2000, 2003). In total, 28 CBCs and 403 hemi-CBCs were identified among the 16 lineages (Supplementary Table 2). Neither CBCs nor hemi-CBCs were present between sequences from the same numbered clade. By contrast, the number of (hemi-)CBCs varied from 1 to 7 between the different clades. Three CBC sites were revealed in ITS secondary structure transcripts: in helices I and II of ITS1, and in helix I of ITS2. In all three cases, the base change U:A – C:G was detected. In ITS1, six hemi-CBC sites could be identified as well. These were recognized mainly in helices I and II. Seven hemi-CBC sites were located in the stem region of helices I, II and III of ITS2. The highest number of hemi-CBCs (7) was determined between clades #1–3, #1–9, #3–8, and #8–9, whereas the highest number of CBCs (2) occurred between clades #5–11, #9–11, and #9–14 (Supplementary Table 2). The closely related *T. glomerata* and *T. irregularis* (clades #1 and #2) differed in one hemi-CBC occurring in helix II of ITS2 (Fig. 4B). In *A. phycobiontica* (clade #15) and clade #16, one hemi-CBC was recorded in helix I of ITS2 (Fig. 4B). A single hemi-CBC was determined between clade #4 and clades #13, 14 and 16, as well. Similarly, the clades #4 and #6 were distinguished by single CBC and no hemi-CBC.

4. Discussion

4.1. Phylogenetic inference

ITS and partial actin sequences were used to infer phylogenetic relationships among several lineages of *Trebouxia* algae belonging to *Asterochloris* sensu Tschermak-Woess (1989). The loci studied revealed considerable differences in their evolutionary dynamics as well as sequence variation (Table 1 and Fig. 2A). According to previously published data (Kroken and Taylor, 2000; Nelsen and Gargas, 2006, 2008), the actin sequences show much

greater variation, and the phylogenies yield strong resolution and support. Accordingly, this study also confirms the improved resolution using actin over ITS sequences (compare Supplementary Figs 1 and 2). Moreover, the ITS locus cannot accurately resolve the closely related lineages, e.g. *T. glomerata* and *T. irregularis*.

In general, both single-locus phylogenies showed an absolute congruence in strain placement into highly supported clades. Since the actin phylogeny receives an almost twofold higher average support of all nodes, we recommend preferably using the actin locus for resolving phylogenies in *Asterochloris*, though ITS appears to remain applicable to determine the species membership of studied organisms. Although using statistical support for branches as the only criterion for choosing the better phylogeny is somewhat illegitimate (Gontcharov et al., 2004), the superiority of actin analysis is supported by several independent morphological characters. For example, the ultrastructural similarity of *T. erici* UTEX 911 and *T. magna* UTEX 902 (Friedl 1989a) was corroborated by close relation of these species only in the actin phylogram (compare Supplementary Figs 1 and 2).

Although the position of some clades obviously varied in the phylograms inferred from the ITS rDNA and actin data, the overall topological differences did not represent significant conflict, nor were any well-supported relationships in conflict between phylogenies, therefore allowing combination of both loci into a single analysis. The combined ITS + actin phylogenetic analysis was found to represent an improvement of the actin phylogram, yielding a better resolved tree with higher support of internal branches. The combined alignment exhibited the highest proportion of fully resolved quartets (79%) and the smallest percentage of fully unresolved quartets (13.8%). The concatenated phylogeny ascertained three highly supported clades (Fig. 3). Clade A corresponds well with Clade I *sensu* Piercey-Normore and DePriest (2001), except for the additional inclusion of *T. erici* and *T. magna*. The close relationship of these two species, as well as the evident position of *T. erici* as a close relative of other strains, rule out use of *T. erici* as an outgroup, although frequently applied (Beiggi and Piercey-Normore, 2007; Cordeiro et al., 2005; Nelsen and Gargas, 2008; Piercey-Normore and DePriest, 2001; Yahr et al., 2004, 2006). For the reason that incorrectly rooted trees may result in misleading phylogenetic and taxonomic inferences (Leliaert et al., 2007), we recommend the use of either unrooted or midpoint rooted phylogenies, until the outgroup is detected.

It is well established that choosing a suitable model of sequence evolution for the loci analyzed is crucial for obtaining reliable phylogenies (Verbruggen and Theriot, 2008). Even if the dataset is composed of a single (e.g. nuclear) marker, separate groups of characters could

evolve under different evolutionary processes. For example, when an alignment is composed of protein-coding sequences, each codon position may be evolving differently; moreover the third codon position may be potentially affected by higher substitutional saturation. Therefore, partitioning the data into a number of separate regions and allowing each region to have its own set of model parameters is expected to result in better fit of the model to the data. In our BI analyses, we used the partitioning strategy not only to separate particular regions within both markers (ITS1, 5.8 rRNA, and ITS2 in ITS dataset; two introns and exon in actin dataset), but also to differentiate groups of characters within these regions (see Section *2.4*). In the ITS dataset, region partitioning yielded better node resolution, whereas the division into stem and loop fragments did not improve the phylogenetic signal (Table 2). No partitioning strategy led to an increase of node resolution in the actin dataset. In the concatenated dataset, slight increase of node resolution was equally induced by region and doublet + C_{123} partitioning. In all datasets, however, parallel division into region partitions and stem/loop fragments (for ITS dataset) or codon positions (for actin dataset) did not further improve the phylogenetic signal. All the above-mentioned results point to the importance of using partitioned datasets when inferring phylogenies based solely on ITS data.

4.2. Diversity of Asterochloris

By investigating both morphological and molecular data, 16 distinct lineages were identified as belonging to the genus *Asterochloris*. However, because only seven lineages could be affiliated with some of described species, this genus apparently still contains many undescribed species. The concatenated ITS + actin phylogram indicates nine well-supported lineages that could represent new species (Fig. 3). However, the presented phylogram gives us only partial insight into the recognized genetic variability of *Asterochloris*, as the actin data have been used only twice to date (Nelsen and Gargas, 2006, 2008). Conversely, ITS sequences have been frequently utilized in many studies of *Asterochloris*, and could help us in accurate species recognition. For example, a lineage of North and Central American lichen photobionts (clade #9) is described in a number of papers (Cordeiro et al., 2005 – Clade IIc; Nelsen and Gargas, 2006 – photobionts from *Pilophorus* cf. *cereolus* and *Stereocaulon* sp.; Nelsen and Gargas, 2008 – the uppermost lineage in Clade II; Piercey-Normore and DePriest, 2001 – the uppermost lineage in Clade II; Yahr et al., 2004, 2006 – Clade IIa). To date, photobionts of this lineage were isolated from 17 species of lichen-forming fungi belonging to five genera. Despite this low specificity, photobionts from clade #9 are known exclusively from the American Continent. Such data could indicate a very interesting geographic

distribution of this lineage. Furthermore, a number of lineages that could represent additional undescribed taxa were revealed by many authors (Beiggi and Piercey-Normore, 2007; Cordeiro et al., 2005; Piercey-Normore and DePriest, 2001; Yahr et al., 2006).

Our results reveal large hidden diversity in the genus *Asterochloris*. Moreover, although previous studies investigated the photobionts from the same mycobiont genera (even the species, e.g. *Lepraria caesioalba*) and ecological groups (i.e. saxicolous, terricolous and epiphytic lichens), we uncovered three new, not yet reported lineages (clades #7, 10, and 14). From 33 isolated photobionts considered in our study, only five of them could be assigned with certainty to previously described species (i.e. *T. glomerata*, *T. irregularis* and *A. phycobiontica*). Obviously, there is a large degree of cryptic diversity which cannot be resolved by morphology. This is one more example of a rapidly growing number of studies showing that in many protist morphospecies, subtle cryptic or pseudocryptic diversity occurs. Several molecular studies recently contributed to the discovery of genetic variability and hidden diversity within several genera of diatoms (Amato et al., 2007; Behnke et al., 2004; Lundholm et al., 2006; Sato et al. 2009; Vanormelingen et al., 2008) or green algae (Kroken and Taylor, 2000; Lewis and Flechtner, 2004; Vanormelingen et al., 2007). Further, our results support the occurrence of substantial cryptic diversity, especially in lichen photobionts, thus far demonstrated in genera *Trebouxia* (Kroken and Taylor, 2000), *Coccomyxa* (Friedl et al., 2007), and *Dictyochloropsis* (Škaloud et al., 2007). Lichen symbiosis may not facilitate the evolution of different morphologies, and therefore, the morphological criteria could be insufficient for revealing the photobiont diversity and making species determinations.

It is important to examine the morphological variability within the *Asterochloris* clades to assess the relevance of traditional morphological characters used for separating species in *Asterochloris*. Afterwards, the proper species concept combining morphological and molecular data could be proposed for the descriptions of additional taxa. The results presented here show that molecular signatures based on unique (hemi-)CBCs in the secondary structure of ITS rRNA could be unambiguously used for delimiting particular *Asterochloris* species.

4.3. Specificity of Asterochloris to lichen-forming fungi

Many morphological and molecular investigations, as well as studies of resynthesis, revealed the specificity of *Asterochloris* algae to certain groups of lichen-forming fungi (e.g. Ahmadjian et al., 1980; Nelsen and Gargas, 2008; Piercey-Normore and DePriest, 2001; Yahr et al. 2004, 2006). *Asterochloris* algae are predominantly associated with lichen-forming

fungi from families Cladoniaceae and Stereocaulaceae (Lecanorales), however, they have been also identified from another fungal taxa within Ascomycota (Supplementary Table 3). Although the occurrence of *Asterochloris* was confirmed molecularly from 11 fungal genera, we are aware of a greater number of photobiont records from only three genera (*Cladonia*, *Lepraria*, and *Stereocaulon*). While *Cladonia* and *Stereocaulon* are associated with all main Asterochloris lineages (A, B, and C), *Lepraria* is selective, and only linked to *Asterochloris* taxa from lineages B and C.

In contrast to the obvious specificity of the entire genus *Asterochloris*, there are particular *Asterochloris* lineages having only low specificity to their mycobionts. According to our results, each algal clade is associated with several fungal species, often even those classified to different genera. In examining several recent molecular investigations focused on *Asterochloris* photobionts (see Introduction for references), its low specificity is made evident. For example, the lineage #1 associates with almost 20 fungal species from genera including: *Anzina*, *Cladonia*, *Diploschistes*, *Hertelidea*, and *Stereocaulon*.

4.4. Taxonomical consequences

The investigation of authentic cultures of *Trebouxia glomerata* (UTEX 895) and *Trebouxia pyriformis* (UTEX 1712) revealed them to be highly similar, considering both molecular markers and morphological characteristics. The formation of pyriform cells having unipolar thickenings, being the diagnostic character of the species (Archibald, 1975), was observed in *T. glomerata* as well. The congruence in morphology, as well as almost identical ITS and actin sequences (99.6% similarity in both loci), lead us to taxonomically join these two species and to establish *Trebouxia pyriformis* as a synonym of *T. glomerata*. Merging of these two species was already suggested by Friedl (1989b) and Piercey-Normore and DePriest (2001), based on their morphological similarity and RAPD amplification patterns, respectively. However, in contrast to another suggestion of Piercey-Normore and DePriest (2001), we retain the species rank of related *T. irregularis,* due to morphological differences in pyrenoid arrangement and zoospore dimensions, as well as dissimilar actin sequences.

Conversely, we revealed distinct differences in two authentic strains of *Trebouxia magna*, i.e. UTEX 902 and UTEX 67. Morphological variations between the strains were already diagnosed by Gärtner (1985b), proposing affiliation of the latter strain to *Trebouxia glomerata*. However, analyses of ITS and actin sequences revealed affiliation of strain UTEX 67 to *T. italiana* (Fig. 3). The strain UTEX 902 is, therefore, proposed to represent the only authentic strain of *T. magna*, given that its morphology well corresponds to the original

species diagnosis made by Archibald (1975). Simultaneously, we established a new type of the species (i.e. lectotype), representing the first drawing of UTEX 902 (originally labeled under the invalid name *´Trebouxia lambii´*, Cult. Coll. 902) in the Ph.D. dissertation of Ahmadjian (1959a).

The repeatedly demonstrated paraphyly of the genus *Trebouxia* (Friedl, 1995; Friedl and Rokitta, 1997; Friedl and Zeltner, 1994; Friedl and Büdel, 2008) calls on the division of *Trebouxia* into two genera. The type species of *Trebouxia* - *T. arboricola* Puymaly - belongs to the lineage with the majority of described species (Beck et al., 1998; Friedl and Rokitta, 1997), and the new generic name *Asterochloris* should be proposed for the other lineage containing *T. erici*, *T. magna* and *Asterochloris phycobiontica*.

Prior to the formal delineation of the genus *Asterochloris*, the taxonomic status of *Pseudotrebouxia* should be resolved. The genus *Pseudotrebouxia* was described by Archibald (1975) to differentiate species of *Trebouxia* having different asexual reproduction. Although the establishment of *Pseudotrebouxia* was later rejected by Gärtner (1985a, b), the description of the genus is essentially similar with the later established *Asterochloris* (Tschermak-Woess, 1980). If the identity of these two genera was demonstrated, the name *Pseudotrebouxia* would have priority over *Asterochloris*. However, the analyses of published ITS sequences show that *Pseudotrebouxia* species belong to the same clade as *Trebouxia* s. str. (Helms et al., 2001, Kroken and Taylor, 2000). Moreover, the ITS sequence of the type species of *Pseudotrebouxia – P. aggregata* UTEX 180 – was recently published by Hauck et al. (2007), confirming that this species is a member of *Trebouxia* s. str.

On the basis of presented comparative molecular investigations, and above-mentioned discussion, we formally transfer six former *Trebouxia* species (*T. erici*, *T. excentrica*, *T. glomerata*, *T. irregularis*, *T. italiana*, and *T. magna*) into *Asterochloris,* along with the establishment of a new genus delimitation. The genus *Asterochloris* is characterized by a unique ITS and actin sequences, as well as by several morphological characteristics (chloroplast morphology, parietal position of chloroplast prior to cell division and frequent aplanospore production). Molecular signatures acquired by the presence of hemi-CBCs in the secondary structure of ITS rRNA (Fig. 4) were added as reliable diagnostic characters for each species.

Asterochloris Tschermak-Woess, 1980; Pl. Syst. Evol. 135, pp. 291, 292 emend. Skaloud et Peksa

Type species: *Asterochloris phycobiontica* Tschermak-Woess, 1980; Pl. Syst. Evol. 135, p. 292.

Emended diagnosis: Single asteroid chloroplast of lobed, crenulate or echinate form. Prior to aplano- and zoosporogenesis, the chloroplast flattens and assumes a parietal position. Asexual reproduction by (16–32–)64–128(–256) aplanospores and zoospores, occasionally by 2–4–8 autospores. Zoospores naked, dorsiventrally flattened, 4–10 μm long × 1.5–4 μm wide, with two apical flagella; stigma present or absent. Photobionts of many lichens (genera *Anzina*, *Cladia*, *Cladonia*, *Diploschistes*, *Lepraria*, *Pilophorus*, *Pycnothelia*, *Stereocaulon*, etc.). Widely distributed, cosmopolitan.

Asterochloris glomerata (Warén) Skaloud et Peksa comb. nov.

Basionym: *Cystococcus glomeratus* Warén, 1920; Reinkulturen von Flechtengonidien, pp. 56–60.

Synonyms: *Trebouxia glomerata* (Warén) Ahmadjian, 1960; Am. J. Bot. 47(8), p. 679, Figs 9, 10, 15. *Trebouxia pyriformis* Archibald, 1975; Phycologia 14(3), pp. 130, 131, Fig. 11.

Lectotype: Warén (1920), Taf. I., Fig. 6 (*hic designatus*).

Epitype: *Asterochloris glomerata* strain UTEX 1712, cryopreserved material deposited at the Culture Collection of Algae at the Charles University in Prague (CAUP, Department of Botany, Charles University in Prague, Benatska 2, CZ–12801, Prague, Czech Republic).

Molecular signatures: Hemi-CBCs in helix I (**C**:G – **U**:G) of the ITS1 and helices I (U:**A** – U:**G**), II (G:**C** – G:**U**; unique!) and III (G:**U** – G:**C**) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

Asterochloris irregularis (Hildreth et Ahmadjian) Skaloud et Peksa comb. nov.

Basionym: *Trebouxia irregularis* Hildreth et Ahmadjian, 1981; Lichenologist 13(1), pp. 82, 83.

Holotype: Hildreth et Ahmadjian (1981), Fig. 2C.

Authentic strain: UTEX 2236

Molecular signatures: Hemi-CBCs in helix I (**C**:G – **U**:G) of the ITS1 and helices I (U:**A** – U:**G**) and III (G:**U** – G:**C**) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

Asterochloris magna (Archibald) Skaloud et Peksa comb. nov.

Basionym: *Trebouxia magna* Archibald, 1975; Phycologia 14(3), p. 130, Fig. 10.

Synonym: *Trebouxia lambii* nomen nudum Ahmadjian, 1959a: 55–57.

Lectotype: Illustration "*Pilophorus acicularis*" A-H inserted between pages 58 and 59 (as *T. lambii* sp. nov.) in Ahmadjian (1959a) (*hic designatus*).

Epitype: *Asterochloris magna* strain UTEX 902, cryopreserved material deposited at the Culture Collection of Algae at the Charles University in Prague (CAUP, Department of Botany, Charles University in Prague, Benatska 2, CZ–12801, Prague, Czech Republic).

Authentic strain: UTEX 902

Molecular signatures: Hemi-CBCs in helices I (G:**C** – G:**U**; unique!) and IV (**U**:G – **C**:G) of the ITS1 and helix I (**U**:G – **C**:G) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

Asterochloris erici (Ahmadjian) Skaloud et Peksa comb. nov.

Basionym: *Trebouxia erici* Ahmadjian, 1960; Am. J. Bot. 47(8), pp. 680, 681.

Lectotype: Ahmadjian (1960), Fig. 6 (*hic designatus*).

Epitype: *Asterochloris erici* strain UTEX 911, cryopreserved material deposited at the Culture Collection of Algae at the Charles University in Prague (CAUP, Department of Botany, Charles University in Prague, Benatska 2, CZ–12801, Prague, Czech Republic).

Authentic strains: UTEX 910, 911, 912

Molecular signatures: Hemi-CBCs in helix I (**C**:G – **U**:G) of the ITS1 and helix I (**U**:G – **C**:G) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

Asterochloris excentrica (Archibald) Skaloud et Peksa comb. nov.

Basionym: *Trebouxia excentrica* Archibald, 1975; Phycologia 14(3), pp. 128, 130.

Lectotype: Archibald (1975), Fig. 7 (*hic designatus*).

Authentic strain: UTEX 1714

Molecular signatures: Hemi-CBCs in helix I (**A**:U – **G**:U; unique!) of the ITS1 and helix I (U:**G** – U:**A**) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

Asterochloris italiana (Archibald) Skaloud et Peksa comb. nov.

Basionym: *Trebouxia italiana* Archibald, 1975; Phycologia 14(3), p. 130, Fig. 9.

Lectotype: Archibald (1975), Fig. 9 (*hic designatus*).

Authentic strain: CCAP 219/5B

Molecular signatures: Hemi-CBCs in helix I (**C**:G – **U**:G) of the ITS1 and helices I (**U**:G – **C**:G) and III (G:**C** – G:**U**; unique!) of the ITS2; as compared to the ITS rRNA secondary transcripts of *A. phycobiontica*.

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Table 1. Specifications, evolutionary models and model parameters obtained for different data sets.

	ITS				Actin				concatenated
	whole partition	ITS1	5.8 rRNA	ITS2	Whole partition	Intron 206	exon	Intron 248	
Alignment length/analyzed	520/520	152/152	166/166	202/202	745/622	262/206	125/125	358/291	1265/1142
Variable sites/parsimony informative sites (in %)	73/48 (14.0/9.2)	36/23 (23.7/15.1)	3/1 (1.8/0.6)	34/24 (16.8/11.9)	392/331 (63.0/53.2)	147/123 (71.4/59.7)	28/26 (22.4/20.8)	217/187 (74.6/64.3)	465/379 (40.7/33.2)
Model estimated[a]	GTR+I+Γ	GTR+Γ	JC	K80+I+Γ	GTR+I+Γ	HKY+Γ	K80+CP_{123}+I	HKY+I	GTR+I+Γ
I, Γ values[b]	0.6208/0.7571	0/1.0842	0/-	0.6150/0.7300	0.2033/4.0201	0/2.2126	0.6372/-	0.1415/-	0.4483/2.4077
Measure of skewness (g_1-value)	-0.62	-0.69	-10.77	-0.50	-0.65	-0.65	-0.56	-0.60	-0.65
I_{ss} statistic (I_{ss} / I_{ss}+c / p-value of 32 taxon data subsets)	0.085/0.694 /p < 0.001	0.032/0.651 / p < 0.001	0.001/0.662 / p < 0.001	0.063/0.663 / p < 0.001	0.210/0.673 / p < 0.001	0.261/0.642 / p = 0.003	0.127/0.656 / p < 0.001	0.350/0.652 / p < 0.001	0.149/0.721 / p < 0.001
Likelihood mapping results (fully resolved/fully unresolved quartets)	54.0/42.5	56.2/40.4	0.3/99.7	49.5/46.1	77.2/18.9	69.5/24.2	58.8/36.0	77.8/18.2	79.0/13.8

[a]Estimated by the the Akaike Information Criterion (AIC) with PAUP/MrModeltest 1.0b
[b]Proportion of invariable sites (I) and gamma distribution shape parameter (Γ) as estimated by PAUP/MrModeltest 1.0b

Table 2. Node resolutions on the BI, ML and MP analyses. Number of nodes receiving high (more than 94% for BI; more than 79% for ML and MP), moderate (50–94% for BI; 50–79% for ML and MP), and low (less than 50% for BI, ML and MP) support are displayed. Explanatory note to the data partitioning: Region – ITS (ITS1, ITS2, 5.8 rRNA) and actin (1st actin intron, 2nd actin intron, actin exon) partitioning; doublet – loop/stem partitioning in ITS; CP_{123} – codon position partitioning in actin-exon.

	ITS	actin	concatenated
BI (no partitions)	10/4/20	24/8/9	25/9/11
BI (region partitioning)	11/6/17	24/9/8	26/7/12
BI (doublet and/or CP_{123} partitioning)	9/7/18	24/9/8	26/8/11
BI (region and doublet and/or CP_{123} partitioning)	11/7/16	24/8/9	26/7/12
ML (GTR+I+Γ)	9/7/18	26/11/4	26/13/6
wMP	9/4/21	24/12/5	26/11/8
Average resolution of all analyses	9.8/5.8/18.4	24.3/9.5/7.2	25.8/9.2/10.0

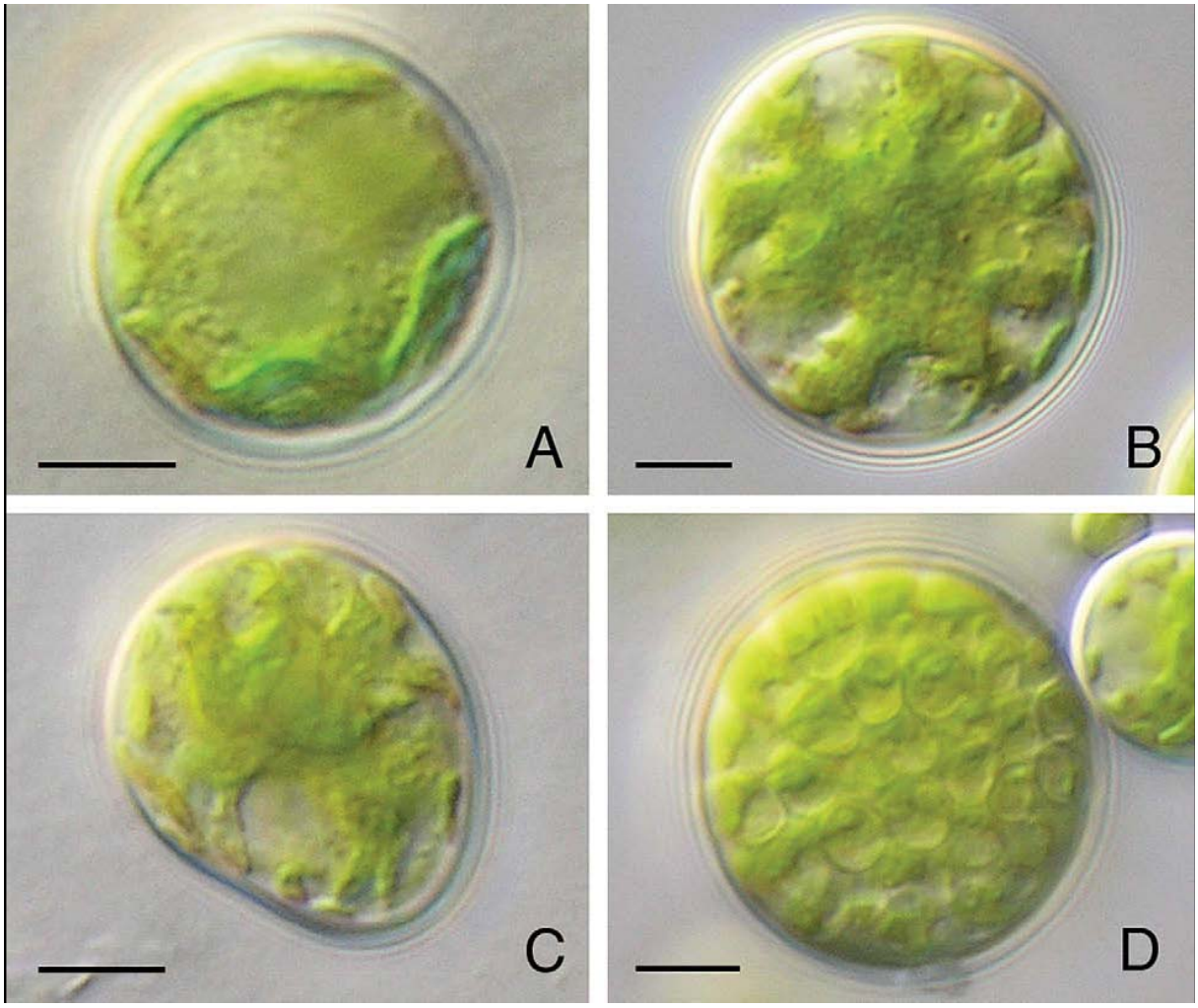

Fig. 1. Light micrographs of *Asterochloris* strains. (A) Parietal chloroplast with smooth, never lobed margins, occuring prior to aplano-, and zoospore production, strain CAUP H1011. This chloroplast stage never occurs prior to autospore formation. (B) Deeply lobed axial chloroplast, strain CAUP H1010. (C) Cell of pyriform shape, strain UTEX 1712. (D) Mature aplanosporangium containing high number of daughter cells (usually 64 or 128), strain UTEX 1712. Scale bar – 5 μm.

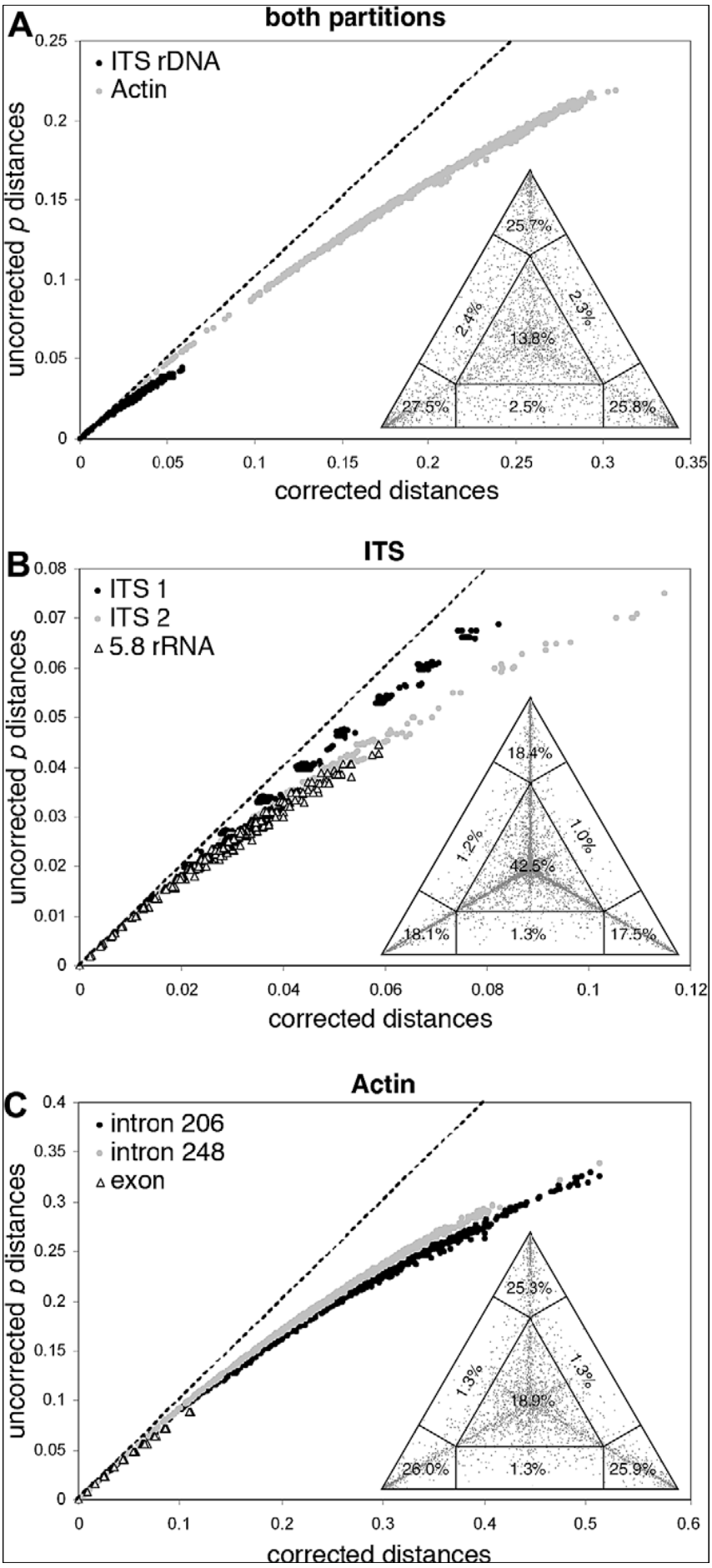

Fig. 2. Analysis of substitutional saturation. The graphs visualize the saturation of the ITS rRNA and actin datasets by plotting ML-corrected distances against uncorrected p-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition (Table 1). (A) Analysis of concatenated alignment. (B) Analysis of ITS rDNA sequences. (C) Analysis of actin sequences. The triangles in the lower right of the graphs illustrate likelihood mapping results. The values in the panels indicate proportion of fully resolved (corners), partially resolved (along the sides), and fully unresolved quartets (in the centre).

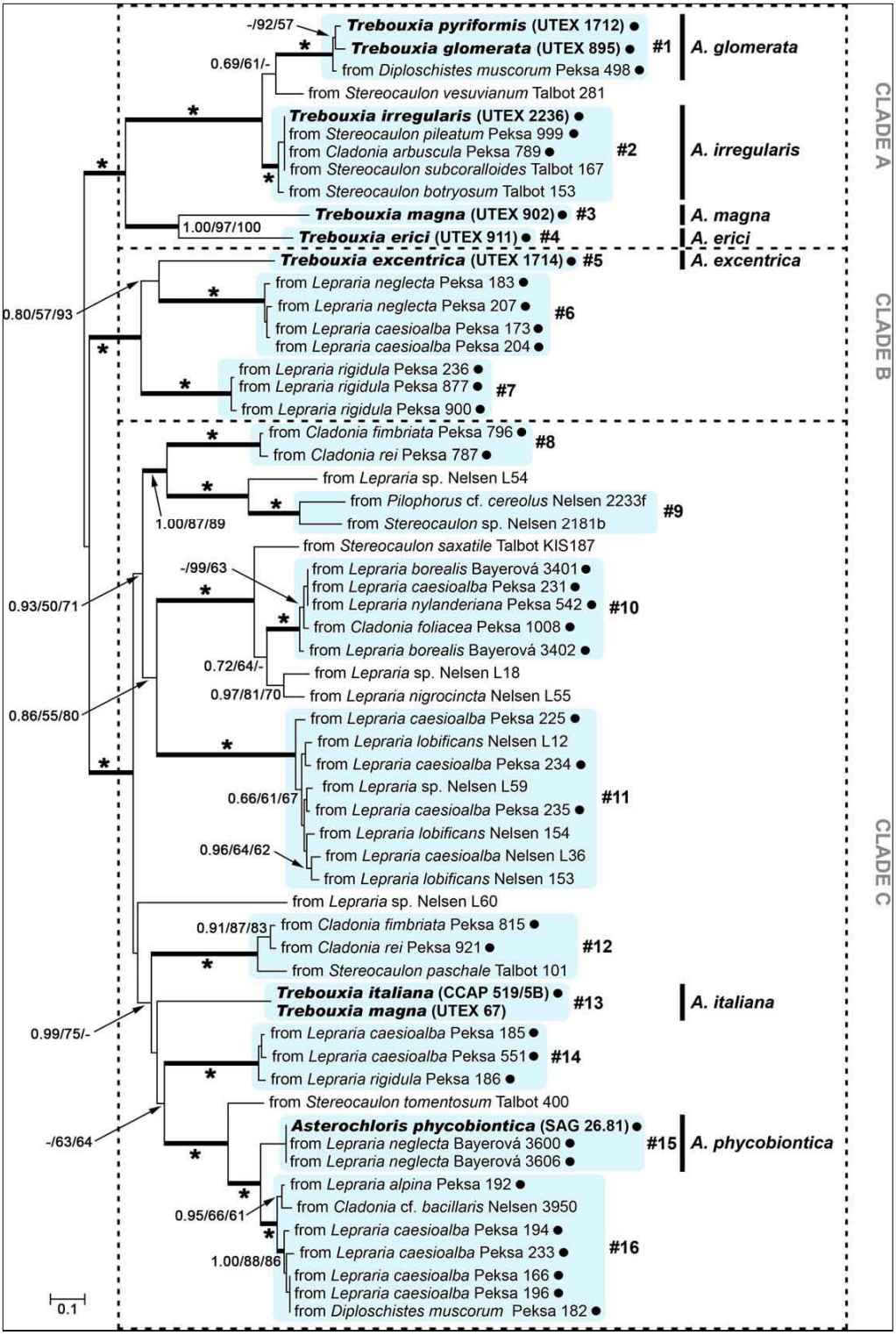

Fig. 3. Unrooted BI analysis based on the combined ITS + actin dataset using a GTR+Γ model for ITS1, K80+I+Γ model for ITS2, and JC model for 5.8 rRNA partition; and a HKY+Γ model for the actin intron 206, HKY+I model for the actin intron 248, and K80+CP_{123}+I model for the actin exon partition. The doublet model is applied for the stem regions in all ITS rDNA partitions. Values at the nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), maximum likelihood bootstrap (in the middle) and maximum parsimony bootstrap (right). Full statistical support (1.00/100/100) is marked with an asterisk. Thick branches represent nodes receiving the highest PP support (1.00). Sequences determined in this study are marked by full circles. Authentic strains of *Asterochloris phycobiontica* and several former *Trebouxia* species are given in bold. Strain affiliation to 16 lineages (#1–16) and three major clades (A–C) is indicated. Scale bar – substitutions per site.

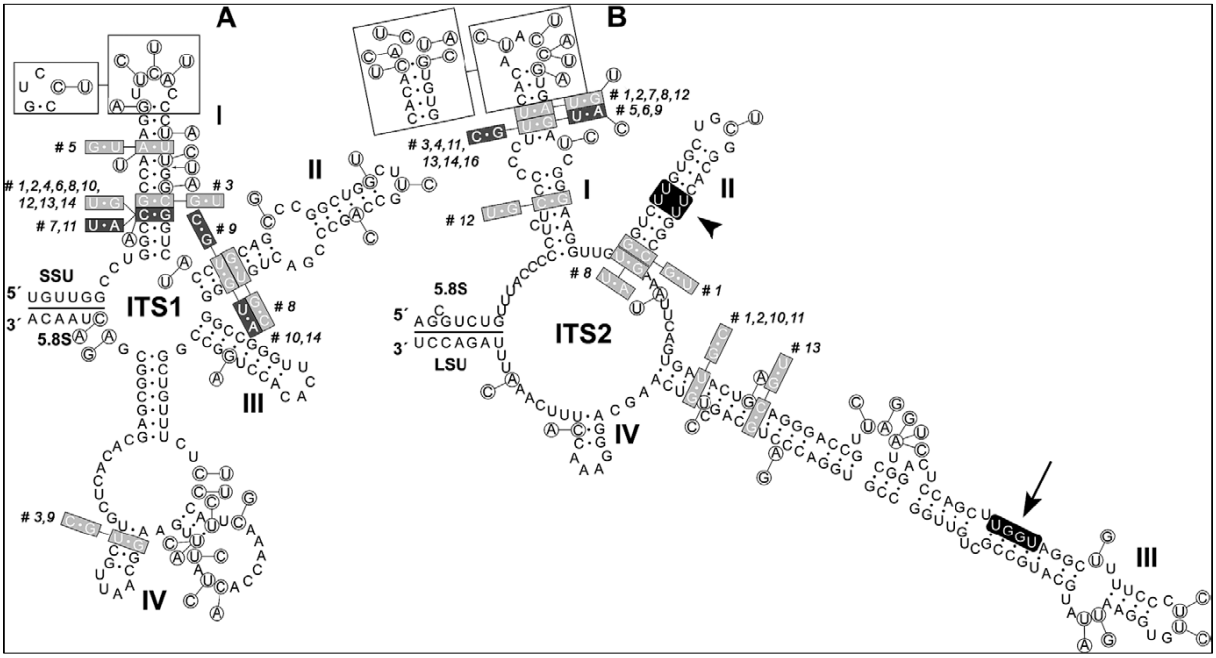

Fig. 4. Predicted secondary structures of the ITS1 (A) and ITS2 (B) transcripts of *Asterochloris phycobiontica* (strain SAG 26.81, EMBL accession number AM900490) derived by comparison among 16 *Asterochloris* lineages. Base changes between the different *Asterochloris* genotypes are indicated: the base pair marked in a dark-grey box indicates compensatory base changes (CBCs); base pairs marked in grey boxes indicate hemi-CBCs; single base changes are marked in circles; changes of the ends in the helices are indicated in large boxes. In ITS2 transcript, the highly conserved U-U mismatches (arrowhead) and UGGU motif (arrow) are highlighted (Schultz et al. 2005). The numbers
next to the boxes (#1–16) specify the *Asterochloris* clades in which the base changes occurred (see Fig. 3).

Supplementary Table 1. *Asterochloris* strains included in this study. Newly obtained sequences are given in bold face.

Taxon and name	Strain	Mycobiont species	Accession numbers	
			ITS	Actin
Asterochloris erici (Ahmadjian) Škaloud et Peksa	UTEX 911	*Cladonia cristatella* Tuck.	AF345440	**AM906018**
Asterochloris excentrica (Archibald) Škaloud et Peksa	UTEX 1714	*Stereocaulon dactylophyllum* Flörke	**AM905993**	**AM906019**
Asterochloris glomerata (Warén) Škaloud et Peksa	UTEX 895	*Stereocaulon evolutoides* (H. Magn.) Frey	AF345382	**AM906024**
Asterochloris glomerata (Warén) Škaloud et Peksa	UTEX 1712	*Cladonia squamosa* (Scop.) Hoffm.	AF345406	**AM906025**
Asterochloris glomerata (Warén) Škaloud et Peksa	DIP 2	*Diploschistes muscorum* (Scop.) R. Sant.	**AM905998**	**AM906026**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	UTEX 2236	*Stereocaulon* sp.	AF345411	**AM906027**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	STER 1	*Stereocaulon pileatum* Ach.	**AM905999**	**AM906028**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	B13	*Cladonia mitis* Sandst.	**AM906000**	**AM906029**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	Talbot 153	*Stereocaulon botryosum* Ach.	DQ229880	DQ229889
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	Talbot 167	*Stereocaulon subcoralloides* Nyl.	DQ229881	DQ229890
Asterochloris italiana (Archibald) Škaloud et Peksa	CCAP 519/5B	„*Xanthoria parietina* (L.) Th. Fr.“	**AM906001**	**AM906030**
Asterochloris italiana (Archibald) Škaloud et Peksa	UTEX 67	*Cladonia* sp.	AF345423	DQ229894
Asterochloris magna (Archibald) Škaloud et Peksa	UTEX 902	*Pilophorus aciculare* (Ach.) Th. Fr.	**AM906012**	**AM906041**
Asterochloris phycobiontica Tschermak-Woess	SAG 26.81	*Anzina carneonivea* (Anzi) Scheid.	AM900490	**AM906042**
Asterochloris phycobiontica Tschermak-Woess	LEP 9	*Lepraria neglecta* (Nyl.) Erichsen	AM900491	**AM906043**

Asterochloris phycobiontica Tschermak-Woess	LEP 7	*Lepraria neglecta* (Nyl.) Erichsen	**AM906013**	**AM906044**
Asterochloris sp. (clade 6)	CAUP H1010, LEP 16	*Lepraria neglecta* (Nyl.) Erichsen	**AM906002**	**AM906031**
Asterochloris sp. (clade 6)	LEP 23	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906003**	**AM906032**
Asterochloris sp. (clade 6)	LEP 25	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906004**	**AM906033**
Asterochloris sp. (clade 6)	LEP 30	*Lepraria neglecta* (Nyl.) Erichsen	**AM906005**	**AM906034**
Asterochloris sp. (clade 7)	CAUP H1013, LEP 6	*Lepraria rigidula* (de Lesd.) Tønsberg	**AM905997**	**AM906023**
Asterochloris sp. (clade 7)	OP 877	*Lepraria rigidula*	**FM955668**	**FM955672**
Asterochloris sp. (clade 7)	OP 900	*Lepraria rigidula*	**FM955669**	**FM955673**
Asterochloris sp. (clade 8)	Backor 04	*Cladonia fimbriata*	**FM945358**	**FM955674**
Asterochloris sp. (clade 8)	Backor 14	*Cladonia subulata*	**FM945380**	**FM955675**
Asterochloris sp. (clade 9)	Nelsen 2233f	*Pilophorus* cf. *cereolus* (Ach.) Th. Fr.	DQ229883	DQ229895
Asterochloris sp. (clade 9)	Nelsen 2181b	*Stereocaulon* sp.	DQ229884	DQ229896
Asterochloris sp. (clade 10)	CAUP H1009, LEP 10	*Lepraria borealis* Lohtander & Tønsberg	AM900492	**AM906045**
Asterochloris sp. (clade 10)	LEP 15	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906014**	**AM906047**
Asterochloris sp. (clade 10)	LEP 34	*Lepraria borealis* Lohtander & Tønsberg	**AM906015**	**AM906048**
Asterochloris sp. (clade 10)	LEP 36	*Lepraria nylanderiana* Kümmerl. & Leuckert	AM900493	**AM906046**
Asterochloris sp. (clade 10)	CLAD 1	*Cladonia foliacea* (Huds.) Willd.	**AM906016**	**AM906049**
Asterochloris sp. (clade 11)	CAUP H1011, LEP 5	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM905995**	**AM906021**
Asterochloris sp. (clade 11)	LEP 4	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM905994**	**AM906020**

Asterochloris sp. (clade 11)	LEP 33	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM905996**	**AM906022**
Asterochloris sp. (clade 11)	Nelsen 3974, 154	*Lepraria lobificans* Nyl.	DQ229877	DQ229898
Asterochloris sp. (clade 11)	Nelsen 3966, L36	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	EU008664	EU008697
Asterochloris sp. (clade 11)	Nelsen 3960, L12	*Lepraria lobificans* Nyl.	EU008675	EU008704
Asterochloris sp. (clade 11)	Nelsen 3973, 153	*Lepraria lobificans* Nyl.	EU008678	EU008707
Asterochloris sp. (clade 11)	Nelsen 2453, L59	*Lepraria* sp.	EU008691	EU008716
Asterochloris sp. (clade 13)	CAUP H1012, LEP 31	*Lepraria rigidula* (de Lesd.) Tønsberg	**AM905992**	**AM906017**
Asterochloris sp. (clade 13)	LEP 32	*Lepraria* sp.	**FM955666**	**FM955670**
Asterochloris sp. (clade 13)	LEP 55	*Lepraria neglecta*	**FM955667**	**FM955671**
Asterochloris sp. (clade 15)	CAUP H1014, LEP 13	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906008**	**AM906037**
Asterochloris sp. (clade 15)	LEP 1	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906006**	**AM906035**
Asterochloris sp. (clade 15)	LEP 2	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906007**	**AM906036**
Asterochloris sp. (clade 15)	LEP 27	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	**AM906009**	**AM906038**
Asterochloris sp. (clade 15)	LEP 28	*Lepraria alpina* (de Lesd.) Tretiach et Baruffo	**AM906010**	**AM906039**
Asterochloris sp. (clade 15)	DIP 1	*Diploschistes muscorum* (Scop.) R. Sant.	**AM906011**	**AM906040**
Asterochloris sp. (clade 15)	Nelsen 3950	*Cladonia cf. bacillaris* (Ach.) Nyl.	DQ229878	DQ229892
Asterochloris sp. (clade 16)	Backor 27	*Cladonia fimbriata*	**FM945359**	**FM955676**
Asterochloris sp. (clade 16)	CLAD 9	*Cladonia rei*	**FM945378**	**FM955677**
Asterochloris sp. (clade 16)	Talbot 101	*Stereocaulon paschale* (L.) Hoffm.	DQ229887	DQ229891

Asterochloris sp.	Nelsen 2166a, L18	*Lepraria* sp.	EU008687	EU008714
Asterochloris sp.	Nelsen 2211a, L54	*Lepraria* sp.	EU008684	EU008711
Asterochloris sp.	Nelsen 2585, L60	*Lepraria* sp.	EU008690	EU008715
Asterochloris sp.	Nelsen 3637b, L55	*Lepraria nigrocincta* Diederich, Sérus. et Aptroot	EU008681	EU008710
Asterochloris sp.	Talbot 281	*Stereocaulon vesuvianum* Pers.	DQ229885	DQ229888
Asterochloris sp.	Talbot 400	*Stereocaulon tomentosum* Th. Fr.	DQ229882	DQ229893
Asterochloris sp.	Talbot KIS 187	*Stereocaulon saxatile* H. Magn.	DQ229886	DQ229897

Supplementary Table 2. Number of CBCs (upper right corner) and hemi-CBCs (lower left corner) occurred among 16 *Asterochloris* lineages, as revealed by the comparison of ITS1 and ITS2 secondary structure transcripts (Fig. 5).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	7	6		0	1	1	1	0	1	0	1	0	0	0	0	0
4	4	3	3		0	1	0	0	1	0	0	0	0	0	0	0
5	6	5	3	4		0	1	0	0	0	2	0	1	1	0	1
6	4	3	3	0	2		0	0	0	0	1	0	1	1	0	1
7	3	2	4	3	3	3		0	1	0	0	0	0	0	1	1
8	4	3	7	4	6	4	3		0	0	0	0	0	0	0	0
9	7	6	2	3	3	3	4	7		1	2	0	1	2	0	1
10	3	2	6	3	5	2	4	4	4		0	0	0	0	0	0
11	4	3	3	3	2	2	3	6	3	3		0	0	0	0	1
12	3	2	6	3	5	3	2	3	6	4	5		0	0	0	0
13	5	4	4	1	3	1	4	5	4	4	3	4		0	0	0
14	5	4	4	1	3	1	4	5	2	2	3	4	2		0	0
15	4	3	3	2	2	2	1	4	3	3	3	3	3	3		0
16	5	4	2	1	1	1	2	5	2	4	1	4	2	2	1	

Supplementary Table 3. List of taxa of lichen-forming fungi associated with *Asterochloris*. The genera where identity of *Asterochloris* was confirmed by molecular data are given in bold. Number of species means number of particular fungal species whose photobiont identity is known.

Genus	Classification	Number of species	References
Anzina	Ostropomycetidae inc. sed., Lecanoromycetes	1	20, 24
Calicium	Physciaceae, Teloschistales, Lecanoromycetes	1	21
Cetraria	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Cladia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	18, 20, 23
Cladonia	Cladoniaceae, Lecanorales, Lecanoromycetes	69	1, 2, 3, 4, 7, 8, 9, 11, 14, 15, 16, 17, 19, 20, 25, 26, 27
Clauzadea	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	2
Cyphelium	Physciaceae, Teloschistales, Lecanoromycetes	1	21
Diploschistes	Thelotremataceae, Ostropales, Lecanoromycetes	2	11, 13, 27
Evernia	Parmeliaceae, Lecanorales, Lecanoromycetes	2	16
Hypogymnia	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Hypotrachyna	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Chaenotheca	Coniocybaceae inc. sed., Ascomycota	1	21
Lecidea s.l.	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	22
Lepraria	Stereocaulaceae, Lecanorales, Lecanoromycetes	12	2, 5, 10, 17, 18, 27
Parmelia s.l.	Parmeliaceae, Lecanorales, Lecanoromycetes	3	12
Parmeliopsis	Parmeliaceae, Lecanorales, Lecanoromycetes	2	12
Pilophorus	Cladoniaceae, Lecanorales, Lecanoromycetes	2	3, 17, 18
Platismatia	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Porpidia	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	2	2, 15
Psora	Psoraceae, Lecanorales, Lecanoromycetes	1	22
Pycnothelia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	20
Squamarina	Stereocaulaceae, Lecanorales, Lecanoromycetes	2	6, 22
Stereocaulon	Stereocaulaceae, Lecanorales, Lecanoromycetes	8	1, 3, 15, 17, 20, 27
Usnea	Parmeliaceae, Lecanorales, Lecanoromycetes	3	16
Verrucaria	Verrucariaceae, Verrucariales, Chaetothyriomycetes	1	8
Xanthoria	Teloschistaceae, Teloschistales, Lecanoromycetes	1	3

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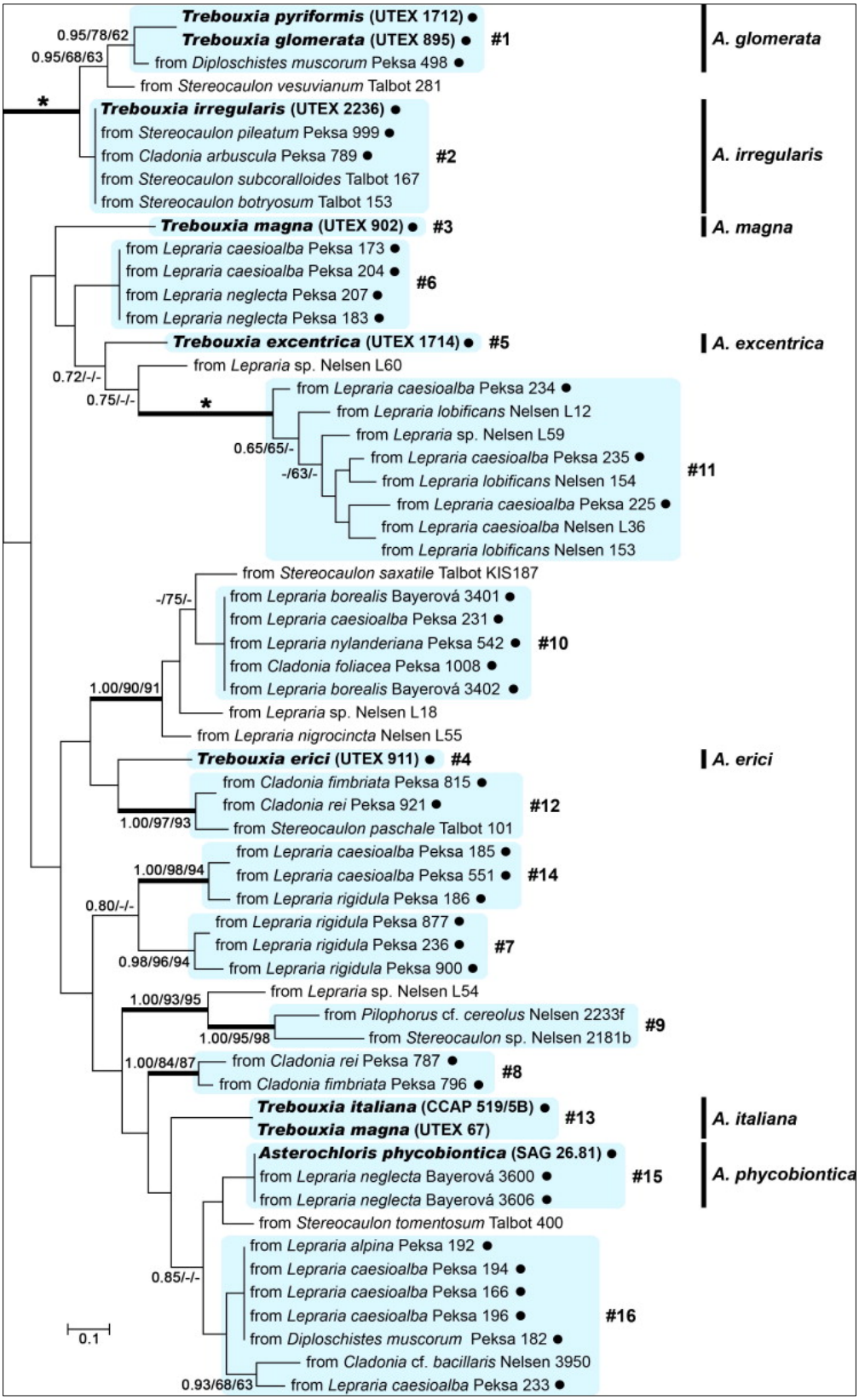
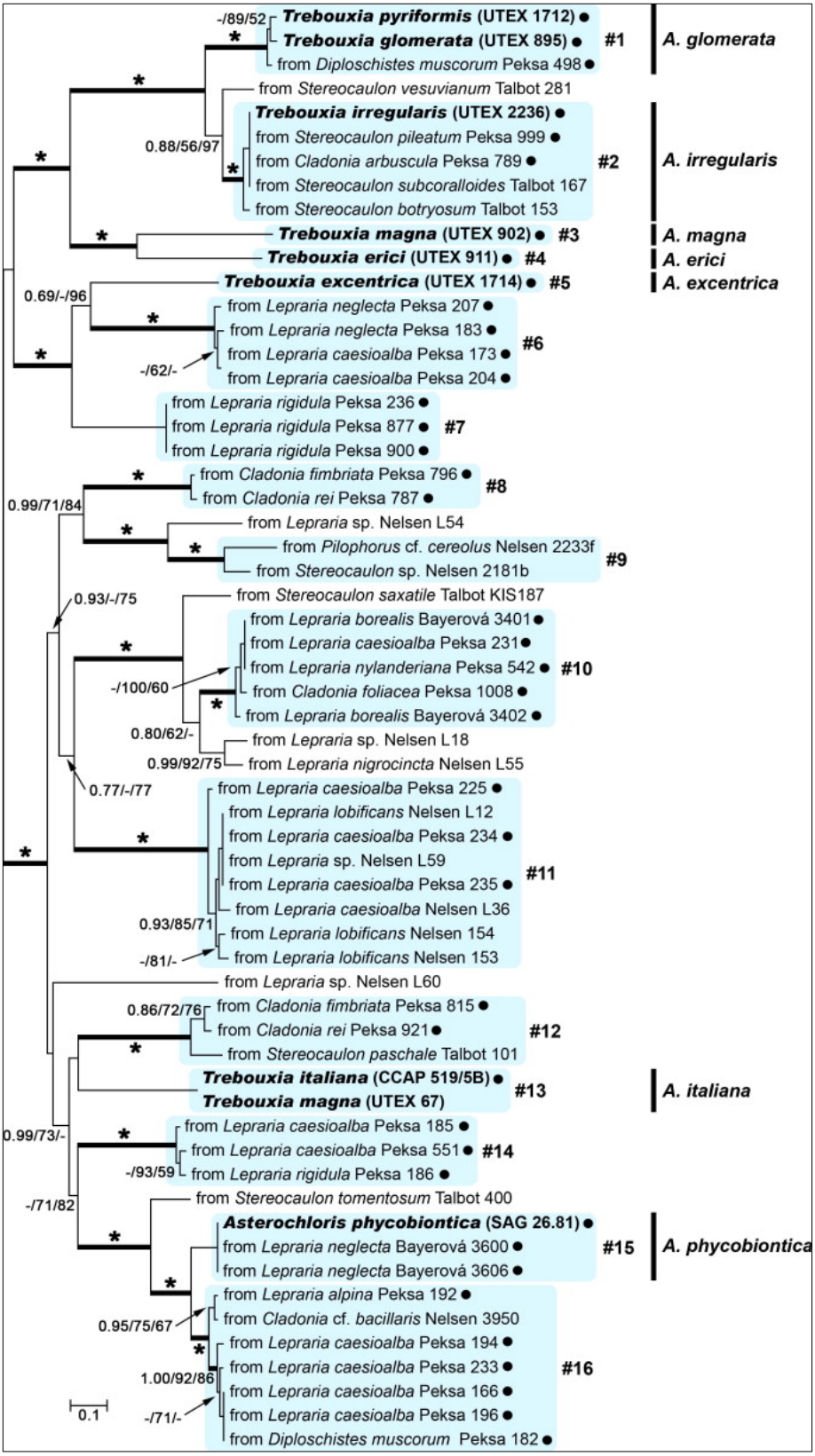


Supplementary Fig. 1. Unrooted BI analysis based on partitioned ITS dataset using a GTR+Γ model for ITS1, K80+I+Γ model for ITS2, and JC model for 5.8 rRNA partition. The doublet model is applied for the stem regions in all partitions. Values at the nodes indicate statistical support estimated by three methods - maximum likelihood bootstrap (top left), maximum parsimony bootstrap (top right) and MrBayes posterior node probability (lower). Thick branches represent nodes receiving the highest PP support (1.00). ITS sequences determined in this study are given in bold face. Scale bar – substitutions per site.



Supplementary Fig. 2. Unrooted BI analysis based on partitioned actin dataset using a HKY+Γ model for the actin intron 206, HKY+I model for the actin intron 248, and K80+CP_{123}+I model for the exon partition. 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). Full statistical support (100/100/1.00) is marked with an asterisk. Thick branches represent nodes receiving the highest PP support (1.00). Actin sequences determined in this study are given in bold face. Scale bar – substitutions per site.

9 Conclusion

The main results of the thesis can be outlined as follows:

- Almost 240 new photobiont sequences from 154 lichen specimens were produced. Eight species of lichen-forming fungi were investigated that have not been analysed for their photobionts to date.
- Many new lineages (species) of lichenized algae were revealed during the investigation of lichen taxa poorly examined for their photobionts (*Lepraria* species).
- Under specific conditions, the photobionts can very easily disengage from the lichen thallus and prosper as free-living algae, providing the possibility to obtain axenic photobiont cultures using simple „fragment method“.
- Significant differences were revealed in chloroplast structure of lichenized and cultured (free-living) algae. Photobionts exhibited gradual changes in chloroplast shape and structure during the successive phases of their liberation from lichen thalli.
- All studied fungi were strictly specific to their photobionts at the generic level, however, at the level of species, their specificity was rather low.
- The particular photobiont lineages were found to be frequently shared among closely related fungi (within one genus or intrageneric group), but also among unrelated fungi within a local lichen community or in geographically distant lichens. The research of *Lepraria* confirmed the existence of intensive photobiont sharing among completely sterile lichen fungi.
- The investigation of specificity in various lichen associations confirmed that the high specificity of both partners is rather uncommon.
- During the study of lichenized algae in *Lepraria*, some photobiont lineages exhibited specific environmental requirements (occuring in ecologically similar lichens). In contrast, a study of a terricolous lichen community from metal-polluted habitats revealed photobionts with very broad ecological amplitudes.
- The rain and sun exposure were the most significant environmental factors, clearly distinguishing the *Asterochloris* lineages. The photobionts from ombrophobic and ombrophilic lichens were clustered in completely distinct clades. Moreover, two *Asterochloris* clades were obviously differentiated on the basis of their substrate and climatic preferences.

- The euryecious photobionts probably very often participate in the colonization of new habitats by pioneer lichens. Simultaneously, low specificity as well as selectivity of lichen-forming fungi were revealed in several taxa of pioneer lichens that can therefore serve as an important photobiont transferring system.

10 Appendix 1

List of lichen-forming fungi associated with Asterochloris

During my studies, I compiled the list of lichen-forming fungi which have been reported as associating with *Asterochloris* algae. The list specifies and extends Supplementary Table 3 in *Paper 5*.

In total, 143 fungal species (excluding several incompletely determined specimens – "sp.") from 31 genera have been found to contain *Asterochloris* as their photobiont. The majority of them belong to three genera from Cladoniaceae and Stereocaulaceae (Lecanorales): *Cladonia* (80 completely determined species), *Lepraria* (15 species) and *Stereocaulon* (8 species). In these genera, identity of the photobiont has been confirmed by DNA sequencing, similar to *Anzina*, *Cladia*, *Diploschistes*, *Hertelidea*, *Hymenelia*, *Ochrolechia*, *Pilophorus*, *Psora*, *Pycnothelia*, *Romjularia* and *Squamarina*. The remaining records of the occurrence of *Asterochloris* in lichens from Parmeliaceae, Physciaceae, Porpidiaceae and Teloschistaceae are questionable, because these taxa should be exlusively associated with the genus *Trebouxia*, as confirmed by many molecular studies focused on taxa from these groups (e.g. Doering & Piercey-Normore 2009, Hauck et al. 2007, Nyati 2007, Piercey-Normore 2006, Tibell & Beck 2001 – see chapter 6 References).

Thus, there exist 14 genera of lichen fungi in which *Asterochloris* has been undoubtly recognized (using molecular methods). The questionable records are in red.

Explanatory notes:

Genus, species:

"*Anzina (27 - Varicellaria)*" – current name (in reference number 27, the former name *Varicellaria* is used).

Records:

A. – *Asterochloris*, *C.* – *Cystococcus*, *T.* – *Trebouxia*. The taxa of *Cystococcus* are mentioned with whole nomenclature.

"15 (*T. excentrica*), 17" – in reference number 15, the photobiont was morphological determined as *T. excentrica*; in reference number 17, the phototobiont was investigated using molecular methods, without morphological identification.

" 27 (*A. phycobiontica*, SAG 26.81 - seq. 24)" - in reference number 27, the photobiont was morphological determined as *A. phycobiontica*; the respective strain SAG 26.81 was firstly sequenced in reference number 24.

Notes:

* The affiliation of Culberson´s unnamed lichen to *Lepraria lobificans* was confirmed by Laundon (1992: *Lepraria* in the British Isles. Lichenologist 24: 315–350).

** *C. cohaerens* Chod. has not been confirmed as belonging to *Asterochloris*. However, Warén (1920) included *C. cohaerens* to subgenus *Eleuterococcus* beside the species such as *Cystococcus glomeratus* (*Asterochloris glomerata*), *C. irregularis* (*A. irregularis*) and *C. Cladoniae*. Accordingly, based on the description and illustration of Chodat (1913), I feel its affiliation to *Asterochloris*.

Genus	species	Records
Anzina (27 - *Varicellaria*)	*carneonivea*	21, 27 (*A. phycobiontica*, SAG 26.81 - seq. 24)
Calicium	*abietinum* var. *minutum* (Körb.) Keissl.	22 (*C. Cladoniae endiviaefoliae*)
Cetraria	*islandica*	12 (*T. irregularis*)
Chaenotheca	*chrysocephala*	22 (*C. Cladoniae furcatae*, *C. Cladoniae endiviaefoliae*)
Cladia	*aggregata*	7, 18, 21, 25 (*T. excentrica*), 26 (*T. erici, T. glomerata*)
Cladonia	*arbuscula*	21, 30, 40
Cladonia	*atlantica*	21
Cladonia	*bacillaris* (17 - cf. *bacillaris*)	2 (*T. excentrica*), 15 (*T. excentrica*), 17, 31 (*T. erici*, *T. excentrica*)
Cladonia	*bellidiflora*	16 (*T. pyriformis*), 21
Cladonia	*boryi*	15 (*T. glomerata*)
Cladonia	*botrytes*	21
Cladonia	*caespiticia*	17
Cladonia	*calycantha*	31 (*T. excentrica*)
Cladonia	*capitellata*	21
Cladonia	*caroliniana s.str.*	21
Cladonia	*cenotea*	21
Cladonia	*chlorophaea*	7, 14 (*T. pyriformis*), 21, 31 (*T. glomerata*), 36
Cladonia	*coccifera*	16 (*T. pyriformis*), 21, 28 (*C. glomeratus*), 42
Cladonia	*confusa*	9
Cladonia	*coniocraea*	7, 16 (*T. erici*), 42
Cladonia	*cornuta*	7, 28 (*C. glomeratus*)
Cladonia	*crinita*	9
Cladonia	*crispata*	21, 31 (*T. glomerata*)
Cladonia	*cristatella*	1 (*T. glomerata* - seq. 21), 21
Cladonia	*deformis*	16 (*T. pyriformis*), 28 (*C. glomeratus*), 42
Cladonia	*didyma*	21
Cladonia	*dimorphoclada*	29
Cladonia	*ecmocyna*	7
Cladonia	*evansii*	29
Cladonia	*farinacea*	21
Cladonia	*fimbriata*	7, 8 (*C. irregularis*), 21, 30
Cladonia	*fissidens*	9
Cladonia	*floerkeana*	31 (*T. glomerata*)
Cladonia	*floridana*	21
Cladonia	*foliacea*	19
Cladonia	*furcata*	16 (*T. pyriformis*), 21, 31 (*T. glomerata*), 42
Cladonia	*glauca*	21
Cladonia	*gracilis*	20, 21, 28 (*C. glomeratus*)
Cladonia	*granulans*	31 (*T. excentrica*)
Cladonia	*grayi*	7, 16 (*T. erici*), 21, 37 (*A. sp.*)
Cladonia	*humilis*	31 (*T. erici*, *T. excentrica*), 34 (*T. glomerata*), 42
Cladonia	*krempelhuberi*	31 (*T. erici*, *T. glomerata*)

Cladonia	*leporina*	15 (*T. excentrica*), 29
Cladonia	*macilenta*	28 (*C. glomeratus*), 42
Cladonia	*macrophylodes*	7
Cladonia	*magyarica*	36
Cladonia	*merochlorophaea*	7
Cladonia	*mitis*	4 (*T. irregularis*), 16 (*T. pyriformis*), 42
Cladonia	*monomorpha*	36
Cladonia	*multiformis*	20
Cladonia	*nipponica*	31 (*T. excentrica*)
Cladonia	cf. *novochlorophaea*	42
Cladonia	*ochrochlora*	7, 21, 42
Cladonia	*pachycladodes*	29
Cladonia	*parasitica*	21
Cladonia	*peltasica*	21
Cladonia	*perforata*	9, 29
Cladonia	*pityrea*	16 (*T. pyriformis*)
Cladonia	*pleurota*	16 (*T. pyriformis*), 21, 31 (*T. erici*, *T. glomerata*), 42
Cladonia	*pocillum*	7, 36
Cladonia	*prostrata*	29
Cladonia	*pulviniformis*	21
Cladonia	*pyxidata*	7, 8 (*C. Cladoniae II*),16 (*T. pyriformis*), 21, 36, 42
Cladonia	*ramulosa*	31 (*T. erici*, *T. glomerata*)
Cladonia	*rangiferina*	20, 28 (*C. glomeratus*), 31 (*T. erici*, *T. glomerata*), 42
Cladonia	*rangiformis*	21, 42
Cladonia	*rappii*	21
Cladonia	*rei*	30, 42
Cladonia	*robbinsii*	21
Cladonia	*scabriuscula*	31 (*T. excentrica*), 21
Cladonia	sp.	3 (*T. magna*, UTEX 67 - seq. 17, 21)
Cladonia	sp.	31 (*T. erici*)
Cladonia	sp.	21
Cladonia	sp.	42
Cladonia	*spinea*	21
Cladonia	*squamosa*	3 (*T. pyriformis*, UTEX 1712 - seq. 21), 16 (*T. erici*), 21
Cladonia	*staufferi*	21
Cladonia	*stellaris*	16 (*T. pyriformis*)
Cladonia	*strepsilis*	21
Cladonia	*subsetacea*	29
Cladonia	*subtenuis*	15 (*T. excentrica*), 29, 35
Cladonia	*subulata*	16 (*T. erici*), 21, 42
Cladonia	*symphycarpia* (21 - *symphycarpa*)	21
Cladonia	*turgida*	21
Cladonia	*uncialis*	16 (*T. pyriformis*), 21
Cladonia	*variegata*	21
Cladonia	*verticillaris*	9
Cladonia	*verticillata* (7 - *cervicornis* ssp. *verticillata*)	7, 14 (*T. pyriformis*)
Cladonia	*vulcani*	32 (*T. excentrica*)

Clauzadea (15 - *Lecidea*)	*metzleri*	15 (*T. excentrica*)
Cyphelium	*inquinans*	22 (*C. irregularis*)
Cyphelium	*tigillare*	22 (*C. Cyphelii*)
Diploschistes	*diacapsis*	33 (*T. excentrica*)
Diploschistes	*gypsaceus*	13 (*T. irregularis*)
Diploschistes	*muscorum*	11 (*T. irregularis*), 30, 42
Evernia	*divaricata*	16 (*T. excentrica*)
Evernia	*prunastri*	16 (*T. excentrica*)
Hertelidea	*botryosa*	44
Hymenelia	*coerulea*	41
Hymenelia	*prevostii*	41
Hypogymnia	*krogiae* (12 - *krogii*)	12 (*T. irregularis*)
Hypotrachyna (12 - *Parmelia*)	*taylorensis*	12 (*T. irregularis*)
Lepraria	*alpina*	30, 43
Lepraria	*atrotomentosa*	18
Lepraria	*borealis*	24, 30, 38, 43
Lepraria	*caesioalba* (2 - *zonata*)	2 (*T.*), 5, 18, 30, 43
Lepraria	*casiella*	18
Lepraria	*crassissima*	43
Lepraria	*granulata*	43
Lepraria	*incana*	18, 43
Lepraria	*lobificans*	10 (*T.*)*, 17, 18
Lepraria	*membranacea*	43
Lepraria	*neglecta*	24, 30, 43
Lepraria	*nigrocincta*	18
Lepraria	*nivalis*	18
Lepraria	*nylanderiana* (19, 24 - sp.)	18, 19
Lepraria	*rigidula*	30, 43
Lepraria	sp.	15 (*T. excentrica*)
Lepraria	sp.	43
Lepraria	spp. (several unidentified taxa)	18
Melanelia (12 - *Parmelia*)	*stygia*	12 (*T. irregularis*)
Ochrolechia	*frigida*	38
Parmelia	*discordans* (12 - *omphalodes* subsp. *discordans* (Nyl.) Skult)	12 (*T. irregularis*)
Parmelia	*saxatilis*	12 (*T. irregularis*)
Parmeliopsis	*ambigua*	12 (*T. irregularis*)
Parmeliopsis	*hyperopta*	12 (*T. irregularis*)
Pertusaria	*corallina*	31 (*T. glomerata*)
Pilophorus	*acicularis*	18, 39 (*T. lambii* = *T. italiana* in 3, UTEX 902 - seq. 30)
Pilophorus	*cereolus* (17 - cf. *cereolus*)	17
Platismatia	*glauca*	12 (*T. irregularis*)
Porpidia (15 - *Huillia*)	*albocaerulescens*	15 (*T. glomerata*), 31 (*T. glomerata*)
Porpidia (15 - *Huillia*)	*tuberculosa*	15 (*T. excentrica*)
Psora	*decipiens*	23
Pycnothelia	*papillaria*	21
Romjularia (23 - *Lecidea*)	*lurida*	23
Squamarina	*cartilaginea*	23

Squamarina	*lentigera*	6
Stereocaulon	*botryosum*	17
Stereocaulon	*dactylophyllum*	3 (*T. excentrica*, UTEX 1714 - seq. 21), 21
Stereocaulon	*paschale*	17
Stereocaulon	*pileatum*	1 (*T. glomerata*, UTEX 896, 897; *T. pyriformis* - UTEX 1713 - seq. 21), 30
Stereocaulon	*saxatile* (1 - *evolutoides*)	1 (*T. glomerata*, UTEX 895 - seq. 21), 15 (*T. glomerata*, probably UTEX 895), 17
Stereocaulon	sp.	17
Stereocaulon	sp.	15 (*T. irregularis*, UTEX 2236 - seq. 21)
Stereocaulon	sp.	42
Stereocaulon	*subcoralloides*	17
Stereocaulon	*tomentosum*	17, 42
Stereocaulon	*vesuvianum*	17
Usnea	*filipendula* (16 - *dasypoga*)	16 (*T. excentrica*)
Usnea	*florida*	16 (*T. excentrica*)
Usnea	*rigida*	16 (*T. excentrica*)
Verrucaria	*murina* (8 - *myriocarpa*)	8 (*C. cohaerens*)**
Xanthoria	*parietina*	3 (*T. italiana* - seq. 30)

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11 Appendix 2

List of the lichen specimens investigated (with additional information on associated photobionts)

Explanatory notes:

Mycobiont: **Coll. number** – lichen specimens are deposited in the herbaria PL (The West Bohemian Museum in Pilsen – specimens of O. Peksa) and PRA (The Institute of Botany of the Academy of Science of the Czech Republic – specimens of Š. Slavíková-Bayerová and Z. Palice).

Photobiont: **CC Peksa** – algal culture is deposited in culture collection of O. Peksa; **CAUP** – some of my cultures were duplicated and deposited in CAUP under CAUP collection numbers. **GenBank ITS**, **GenBank actin** – GenBank accession numbers of ITS and actin photobiont sequences.

Paper: a number of paper in which the sample was used.

mycobiont			*photobiont*				*Paper*
Fungal taxon	**Coll. number**	**Origin**	**CC Peksa**	**CAUP**	**GenBank ITS**	**GenBank actin**	
Cetraria aculeata	Peksa 800	Slovakia			FM945343	-	2
Cetraria islandica	Peksa 799	Slovakia			FM945344	-	2
Cetraria islandica	Peksa 812	Slovakia			FM945346	-	2
Cetraria islandica	Peksa 813	Slovakia			FM945345	-	2
Cladonia arbuscula	Peksa 789	Slovakia			FM945347	-	2, 5
Cladonia coccifera	Peksa 588	Czech Rep.			FM945351	-	2
Cladonia coccifera	Peksa 589	Czech Rep.			FM945352	-	2
Cladonia coccifera	Peksa 818	Slovakia			FM945353	-	2
Cladonia coniocraea	Peksa 576	Czech Rep.			FM945354	-	2
Cladonia deformis	Peksa 918	Czech Rep.			FM945357	-	2
Cladonia fimbriata	Peksa 796	Slovakia			FM945358	-	2, 5
Cladonia fimbriata	Peksa 815	Slovakia			FM945359	-	2, 5
Cladonia foliacea	Peksa 1008	Czech Rep.	Clad1	CAUP CRYO9	AM906016	AM906049	1, 5
Cladonia furcata	Peksa 792	Slovakia			FM945361	-	2
Cladonia furcata	Peksa 797	Slovakia			FM945360	-	2
Cladonia furcata	Peksa 811	Slovakia			FM945362	-	2
Cladonia humilis	Peksa 925	Czech Rep.			FM945350	-	2
Cladonia humilis	Peksa 919	Czech Rep.			FM945348-9	-	2
Cladonia macilenta	Peksa 917	Czech Rep.			FM945363	-	2
Cladonia macilenta	Peksa 922	Czech Rep.			FM945364-6	-	2
Cladonia mitis	Peksa 807	Slovakia			FM945368	-	2
Cladonia mitis	Peksa 808	Slovakia			FM945367	-	2
Cladonia cf. *novochlorophaea*	Peksa 798	Slovakia			FM945372	-	2
Cladonia ochrochlora	Peksa 816	Slovakia			FM945369	-	2
Cladonia pleurota	Peksa 810	Slovakia			FM945371	-	2
Cladonia pleurota	Peksa 820	Slovakia			FM945370	-	2
Cladonia pyxidata	Peksa 814	Slovakia			FM945375	-	2
Cladonia pyxidata	Peksa 791	Slovakia			FM945373-4	-	2
Cladonia rangiferina	Peksa 819	Slovakia			FM945376	-	2
Cladonia rangiformis	Peksa 790	Slovakia			FM945377	-	2
Cladonia rei	Peksa 787	Slovakia			FM945380	-	2, 5
Cladonia rei	Peksa 921	Czech Rep.			FM945378	-	2, 5
Cladonia rei	Peksa 794	Slovakia			FM945381-2	-	2
Cladonia rei	Peksa 927	Czech Rep.			FM945355-6	-	2
Cladonia rei	Peksa 809	Slovakia			FM945386-7	-	2
Cladonia sp.	Peksa 920	Czech Rep.			FM945388	-	2
Cladonia subulata	Peksa 924	Czech Rep.			FM945385	-	2
Cladonia subulata	Peksa 916	Czech Rep.			FM945383-4	-	2
Cladonia subulata	Peksa 926	Czech Rep.			FM945379	-	2
Diploschistes muscorum	Peksa 498	Czech Rep.	Dip2	CAUP CRYO15	AM905988	AM906026	5
Diploschistes muscorum	Peksa 923	Czech Rep.			FM945389	-	2
Diploschistes muscorum	Peksa 182	Czech Rep.	Dip1	CAUP CRYO20	AM906011	AM906040	5

Diploschistes muscorum	Peksa 928	Czech Rep.			FM945390	-	2
Hertelidea botryosa	Peksa 1000	Czech Rep.	Hert1		unpubl.	-	-
Lecidea fuscoatra	Peksa 1002	Czech Rep.	Lec1		AM920666	-	1
Lepraria alpina	Peksa 861	Spain			FN556026	-	3
Lepraria alpina	Palice 10772	Norway	Lep60		as FN556023	-	3
Lepraria alpina	Peksa 546	Czech Rep.			FN556023	-	3
Lepraria alpina	Palice 11626	Czech Rep.			as AM906010	-	3
Lepraria alpina	Peksa 192	Czech Rep.	Lep28	CAUP CRYO14	AM906010	AM906039	3, 5
Lepraria alpina	Peksa 860	Spain			FN556035	FN556048	3
Lepraria borealis	Bayerová 3401	Bulgaria	Lep10	CAUP H1009	AM900492	AM906045	3, 4, 5
Lepraria borealis	Bayerová 3402	Bulgaria	Lep34	CAUP CRYO7	AM906015	AM906048	3, 5
Lepraria borealis	Peksa 507	Czech Rep.	Lep40		as AM906015	-	3
Lepraria borealis	Peksa 513	Czech Rep.	Lep39		as AM906015	-	3
Lepraria borealis	Peksa 516	Slovakia	Lep45		as AM906015	-	3
Lepraria borealis	Peksa 532	Czech Rep.	Lep38		as AM906015	-	3
Lepraria borealis	Peksa 543	Czech Rep.	Lep50		as AM906015	-	3
Lepraria borealis	Peksa 554	Sweden	Lep37		as AM906015	-	3
Lepraria borealis	Peksa 865	Czech Rep.			as AM906015	-	3
Lepraria borealis	Peksa 934	Czech Rep.			as AM906015	-	3
Lepraria borealis	Peksa 869	USA, California			FN556039	FN556049	3
Lepraria borealis	Peksa 866	Czech Rep.			FN556044	-	3
Lepraria caesioalba	Peksa 173	Czech Rep.	Lep23	CAUP CRYO3	AM906003	AM906032	3, 5
Lepraria caesioalba	Peksa 204	Czech Rep.	Lep25	CAUP CRYO4	AM906004	AM906033	3, 5
Lepraria caesioalba	Peksa 864	Czech Rep.			as AM906004	-	3
Lepraria caesioalba	Peksa 932	Czech Rep.			as AM906004	-	3
Lepraria caesioalba	Peksa 188	Czech Rep.	Lep14		as AM906014	-	3
Lepraria caesioalba	Peksa 231	Czech Rep.	Lep15	CAUP CRYO6	AM906014	AM906047	3, 5
Lepraria caesioalba	Peksa 512	Czech Rep.			as AM906014	-	3
Lepraria caesioalba	Peksa 225	Romania	Lep33	CAUP CRYO18	AM905996	AM906022	3, 5
Lepraria caesioalba	Peksa 234	Slovakia	Lep4	CAUP CRYO17	AM905994	AM906020	3, 5
Lepraria caesioalba	Peksa 235	Slovakia	Lep5	CAUP H1011	AM905995	AM906021	3, 5
Lepraria caesioalba	Palice 11603	Czech Rep.	ZP11603		as FN556027	-	3
Lepraria caesioalba	Peksa 185	Czech Rep.	Lep32	CAUP CRYO10	FM955666	FM955670	3, 5
Lepraria caesioalba	Peksa 520	Slovakia	Lep49		FN556027	-	3
Lepraria caesioalba	Peksa 551	Czech Rep.	Lep55	CAUP CRYO19	FM955667	FM955671	3, 5
Lepraria caesioalba	Peksa 859	Spain			FN556028	-	3
Lepraria caesioalba	Peksa 930	Czech Rep.			as FN556028	-	3
Lepraria caesioalba	Peksa 929	Czech Rep.			as FN556024	-	3
Lepraria caesioalba	Peksa 931	Czech Rep.			as FN556024	-	3
Lepraria caesioalba	Peksa 961	Czech Rep.	Lep59		FN556024	-	3
Lepraria caesioalba	Peksa 166	Czech Rep.	Lep13	CAUP H1014	AM906008	AM906037	3, 5
Lepraria caesioalba	Peksa 194	Czech Rep.	Lep27	CAUP CRYO13	AM906009	AM906038	3, 5
Lepraria caesioalba	Peksa 196	Czech Rep.	Lep2	CAUP CRYO12	AM906007	AM906036	3, 5

Lepraria caesioalba	Peksa 233	Czech Rep.	Lep1	CAUP CRYO11	AM906006	AM906035	3, 5
Lepraria caesioalba	Peksa 495	Czech Rep.	Lep44		as AM906009	-	3
Lepraria caesioalba	Peksa 503	Czech Rep.			as AM906009	-	3
Lepraria caesioalba	Peksa 509	Czech Rep.			as AM906006	-	3
Lepraria caesioalba	Peksa 527	Czech Rep.	Lep57		as AM906009	-	3
Lepraria caesioalba	Peksa 545	Czech Rep.	Lep42		as AM906009	-	3
Lepraria caesioalba	Peksa 867	USA, California			as AM906009	-	3
Lepraria caesioalba	Peksa 526	Czech Rep.	Lep52		FN556030	-	3
Lepraria caesioalba	Peksa 529	Czech Rep.	Lep54		FN556036	-	3
Lepraria caesioalba	Peksa 868	USA, California			FN556040	-	3
Lepraria caesioalba	Peksa 872	USA, California			FN556041	FN556050	3
Lepraria caesioalba	Peksa 873	USA, California			FN556042	FN556051	3
Lepraria caesioalba	Peksa 874	USA, California			as FN556041	-	3
Lepraria caesioalba	Peksa 544	Czech Rep.			FN556045	-	3
Lepraria crassissima	Peksa 888	Czech Rep.			FN556033	-	3
Lepraria granulata	Bayerová 3324	Bulgaria			FN556029	-	3
Lepraria incana	Peksa 886	Czech Rep.			FN556037	-	3
Lepraria membranacea	Peksa 904	Czech Rep.			FN556038	-	3
Lepraria membranacea	Peksa 905	Czech Rep.			as FN556038	-	3
Lepraria neglecta	Peksa 183	Czech Rep.	Lep16	CAUP H1010	AM906002	AM906031	3, 5
Lepraria neglecta	Peksa 207	Czech Rep.	Lep30	CAUP CRYO5	AM906005	AM906034	3, 5
Lepraria neglecta	Peksa 515	Slovakia	Lep61		as AM906005	-	3
Lepraria neglecta	Peksa 857	Czech Rep.			as AM906005	-	3
Lepraria neglecta	Peksa 933	Czech Rep.			as AM906005	-	3
Lepraria neglecta	Bayerová 3600	Ukraine	Lep7	CAUP CRYO16	AM906013	AM906044	3, 5
Lepraria neglecta	Bayerová 3606	Ukraine	Lep9	CAUP CRYO2	AM900941	AM906043	3, 4, 5
Lepraria neglecta	Peksa 521	Slovakia	Lep46		as AM900941	-	3
Lepraria nylanderiana	Peksa 542	Czech Rep.	Lep36	CAUP CRYO8	AM900493	AM906046	1, 3, 4, 5
Lepraria rigidula	Peksa 236	Czech Rep.	Lep6	CAUP H1013	AM905997	AM906023	3, 5
Lepraria rigidula	Peksa 851	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 852	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 853	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 854	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 856	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 875	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 877	Czech Rep.			FM955668	FM955672	3
Lepraria rigidula	Peksa 878	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 880	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 881	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 883	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 885	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 887	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 889	Czech Rep.			as FM955668	-	3

Lepraria rigidula	Peksa 900	Czech Rep.			FM955669	FM955673	3, 5
Lepraria rigidula	Peksa 901	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 902	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 937	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 940	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 941	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 943	Germany			as FM955668	-	3
Lepraria rigidula	Peksa 944	Germany			as FM955668	-	3
Lepraria rigidula	Peksa 945	Czech Rep.			as FM955668	-	3
Lepraria rigidula	Peksa 946	Slovakia			as FM955668	-	3
Lepraria rigidula	Peksa 948	Slovakia			as FM955668	-	3
Lepraria rigidula	Peksa 956	Czech Rep.			as FM955668		3
Lepraria rigidula	Peksa 938	Czech Rep.			FN556034	-	3
Lepraria rigidula	Peksa 947	Slovakia			as FN556034	-	3
Lepraria rigidula	Peksa 186	Czech Rep.	Lep31	CAUP H1012	AM905992	AM906017	3, 5
Lepraria rigidula	Peksa 855	Czech Rep.			FN556031	FN556047	3
Lepraria rigidula	Peksa 955	Czech Rep.			FN556032	-	3
Lepraria sp.	Peksa 858	Austria			FN556025	-	3
Lepraria sp.	Peksa 870	USA, California			FN556043	FN556052	3
Lepraria sp.	Peksa 871	USA, California			as FN556043	-	3
Lepraria sp.	Peksa 518	Slovakia			FN556046	-	3
Stereocaulon pileatum	Peksa 999	Czech Rep.	Ster1	CAUP CRYO1	AM905999	AM906028	3, 5
Stereocaulon sp.	Peksa 801	Slovakia			FM945392	-	2, 3
Stereocaulon tomentosum	Peksa 786	Slovakia			FM945391	-	2, 3
Xanthoparmelia conspersa	Peksa 1001	Czech Rep.	Par1		AM920667	-	1
Xanthoria parietina	Peksa 596	Czech Rep.	Xant1		unpubl.	-	-

12 Curriculum vitae

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Education:

Since 2003 – postgraduate study at the Dept. of Botany, Faculty of Science, Charles University; theme: Photobiont diversity in lichenized genus *Lepraria*
1998–2003 – undergraduate study – Master level in Biology at the Faculty of Sciences, Charles University in Prague.

Employment:

since 2007 – The West Bohemian Museum in Pilsen – curator of the collection of cryptogams (PL); since 2009 head of the Department of botany
2006 – Charles University in Prague, Faculty of Science, Dept. of Botany – part-time employment in the doctoral project of Czech Science Foundation)
2005 – Administration of the National Park and Protected Landscape Area of Šumava – lichenologist.

Research interests:

Photobionts; diversity and ecology of lichens – lichenofloristics (current interests: lichens of pine forests, shrub communities and industrial deposits).

Editorial boards:

Erica (The West Bohemian Museum in Pilsen; editor-in-chief)

Sborník Západočeského muzea v Plzni (The West Bohemian Museum in Pilsen; member of editorial board)
Bryonora (The Bryological-Lichenological section of the Czech Botanical Society; member of editorial board).

Other activities in lichenology:
External lecturer at Charles University in Prague, Faculty of Science, Dept. of Botany (courses and excursions focused on lichens)
Board of the Bryological and Lichenological Section of the Czech Botanical Society.

13 Publications

Publications impacted

Svoboda D., **Peksa O.** & Veselá J. (2011): Analysis of the species composition of epiphytic lichens in Central European oak forests. – Preslia 83(1): 129–144.

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Malíček J, Palice Z., Bouda F., Czarnota P., Halda J. P., Liška J., Müller A., **Peksa O.**, Svoboda D., Syrovátková L., Vondrák J. & Wagner B. (2008): Lišejníky zaznamenané během 15. jarního setkání Bryologicko-lichenologické sekce ČBS na Sedlčansku [The lichens recorded during 15th Spring Meeting of the Bryological and Lichenological section of the CBS in the Sedlčany region]. – Bryonora 42: 17–30.

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Peksa O. & Škaloud P. (2008): Photobiont diversity in the *Lepraria neglecta* group (Lecanorales, Stereocaulaceae). – In: Nash T. H. & Seaward M. R. D. (eds.), Lichenological abstracts. ***The 6th IAL Symposium and Annual ABLS Meeting: Biology of lichens and Bryophytes***, American Bryological and Lichenological Society and the International Association of Lichenology, Tempe, Arizona, USA, miscellaneous publication No. 2008.

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