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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. 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.

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 heterogeneous, 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,

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1969). However, Gärtner (1985a,b) rejected the establishment of *Pseudotrebouxia* because his observations did not confirm Archibald's conclusions. Moreover, recent molecular data confirm Gartner'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 the 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 result, 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* s. str. (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; Skaloud and Peksa, 2008); deeply lobed chloroplast (Fig. 1B; Ahmadjian, 1959b; Ahmadjian, 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 consists 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

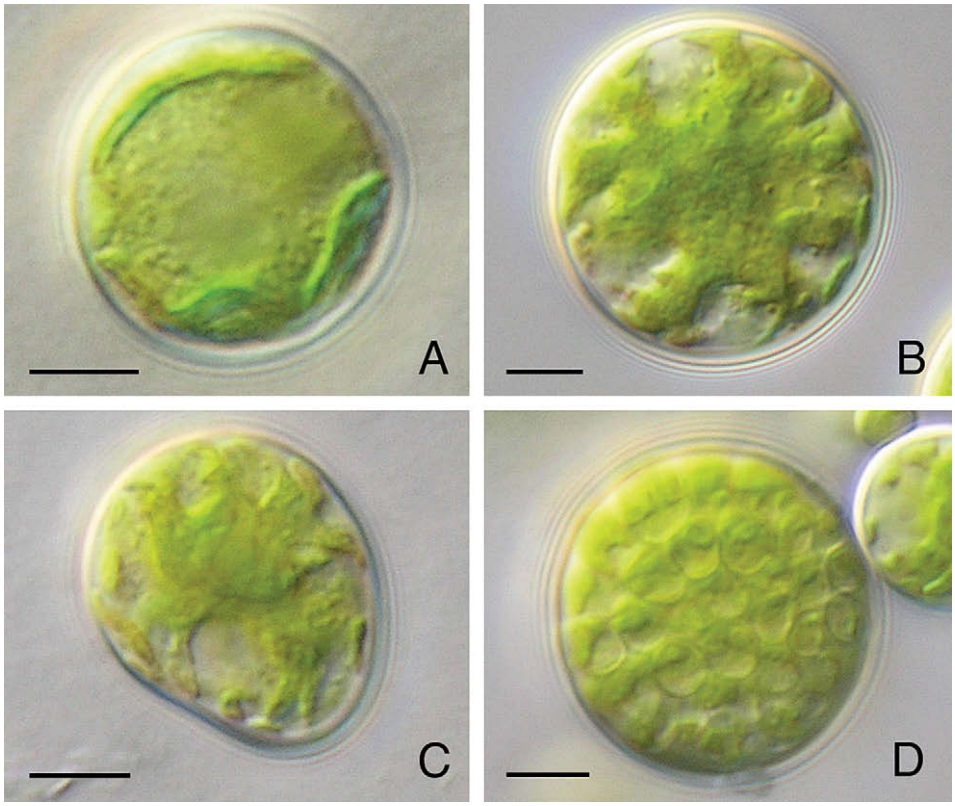

Fig. 1. Light micrographs of *Asterochloris* strains. (A) Parietal chloroplast with smooth, never lobed, margins, occuring prior to aplano-, and zoospore production, strain CAUP H1011. This chloroplast stage never occurs prior to autospore formation. (B) Deeply lobed axial chloroplast, strain CAUP H1010. (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.

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 Š. Slavíková-Bayerová (PRA – Institute of Botany, Academy of Sciences). 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) (1 U/ml), 0.5 μl of $MgCl_2$ (25 mM), 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 Jet-Quick 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 the two following criteria: they represent *Asterochloris* sequences; and both ITS and actin I sequences were available. After deleting identical sequences, the resulting alignments comprised 37 (ITS dataset) and 44 (actin dataset) sequences, or 48 concatenated sequences (concatenated dataset), 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 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 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 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.

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

	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.

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.

Table 2
Node resolutions on the BI, ML and MP analyses. Number of nodes receiving high (more than 94% for BI; more than79% 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

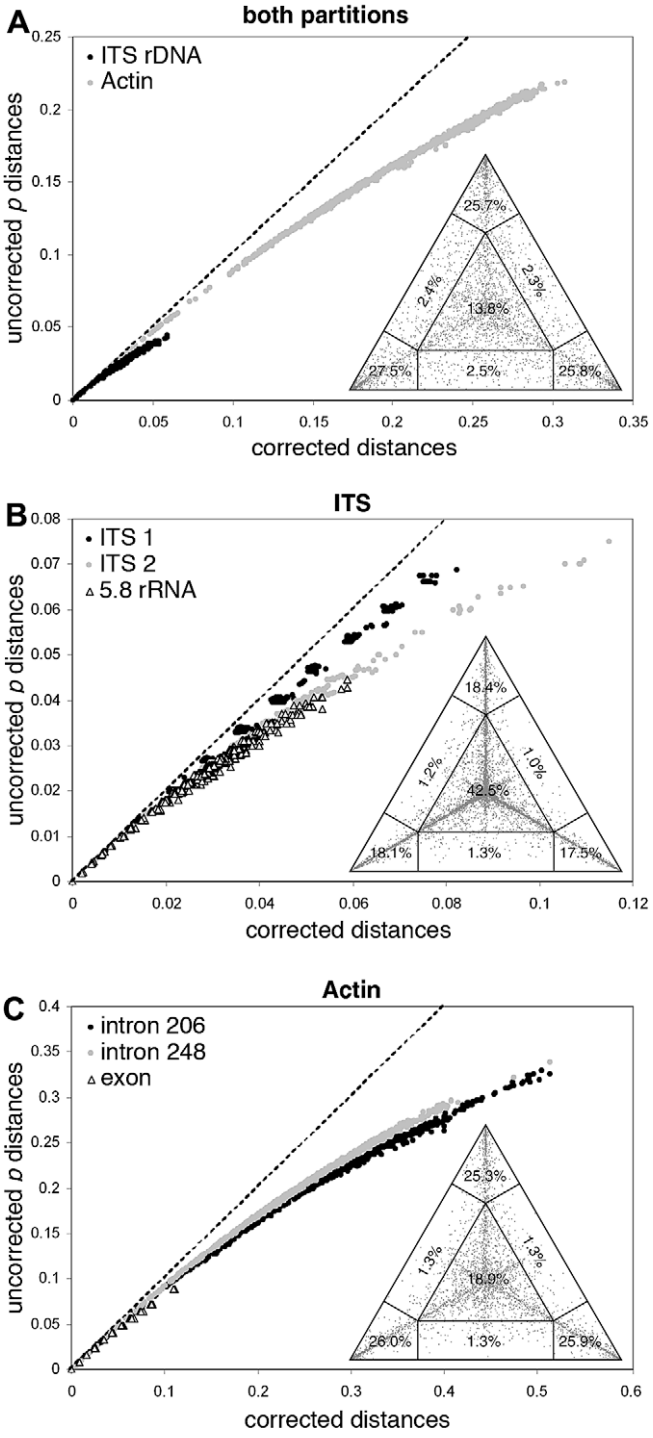

Fig. 2. Analysis of substitutional saturation. The graphs visualize the saturation of the ITS rRNA and actin datasets by plotting ML-corrected distances against uncorrected p-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition (Table 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).

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

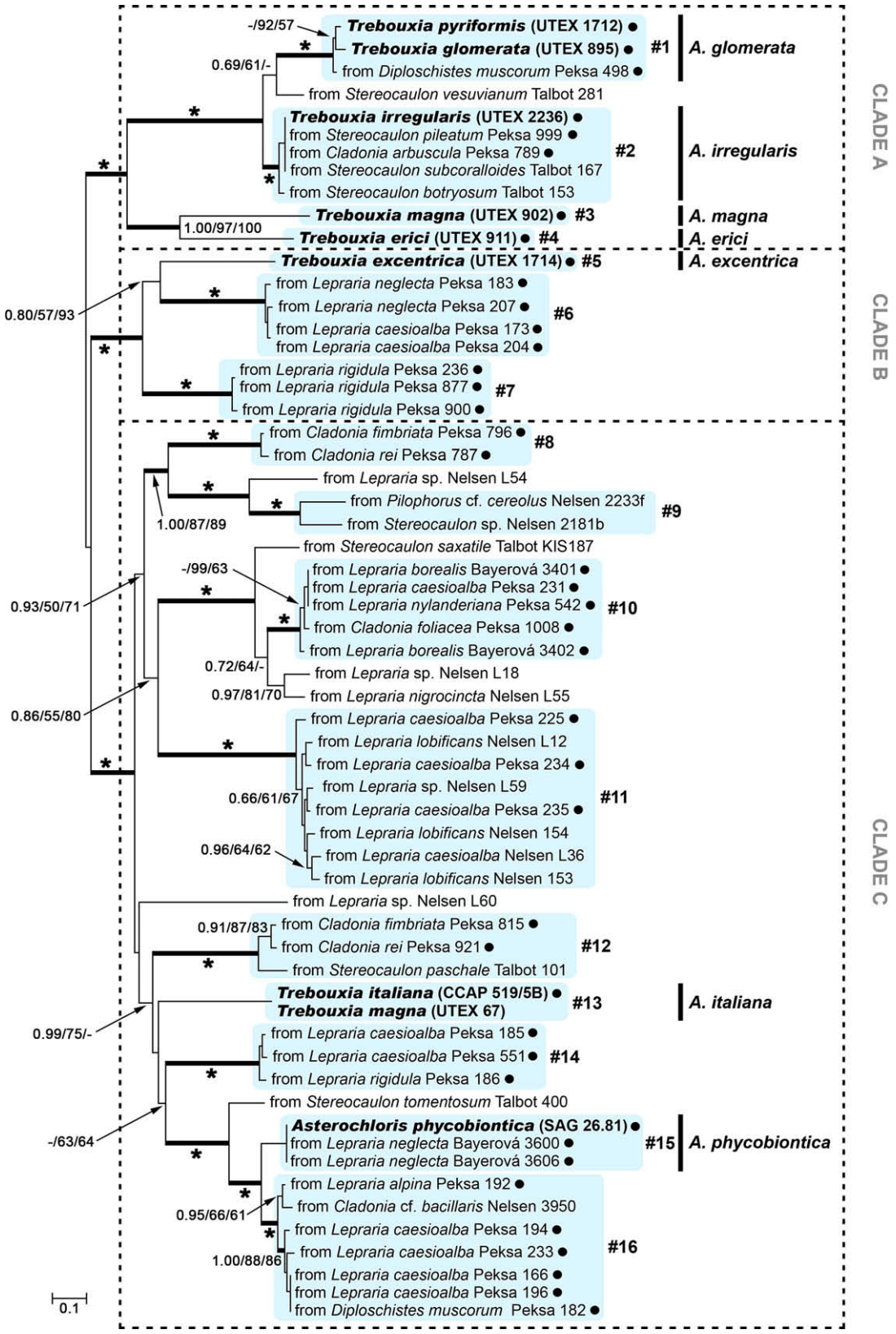

Fig. 3. Unrooted BI analysis based on the combined ITS + actin dataset using a GTR+Γ model for ITS1, K80+I+Γ model for ITS2, and JC model for 5.8 rRNA partition; and a HKY+Γ model for the actin-intron 206, HKY+I model for the actin-intron 248, and K80+CP_{123}+I model for the actin-exon partition. The 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.

Bayesian inference on partitioned datasets (Fig. 3, Supplementary Figs. 1 and 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. 3 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 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 a close relation of these species 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 the use of *T. erici*

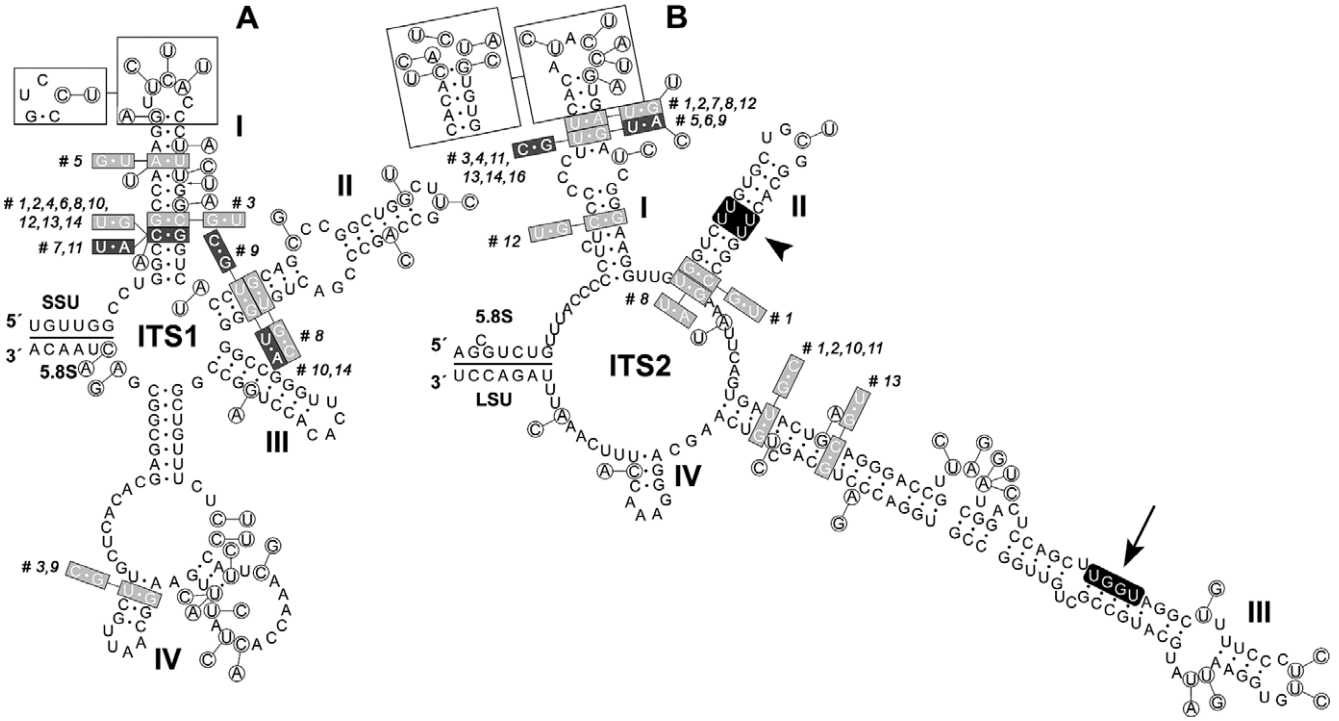

Fig. 4. Predicted secondary structures of the ITS1 (A) and ITS2 (B) transcripts of *Asterochloris phycobiontica* (strain SAG 26.81, EMBL accession number AM900490) derived by comparison among 16 *Asterochloris* lineages. Base changes between the different *Asterochloris* genotypes are indicated: the 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).

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 9 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 5 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 was 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 thirty-three 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. 2008; 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* (Skaloud 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 the 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, 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 pp. 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.09.035.

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Supplementary Table 1. Species and strains of *Asterochloris* included in this study, with strain number (if strain exist), name of associated mycobiont species, number of herbarium specimen and GenBank accession numbers of algal sequences (newly obtained sequences are given in bold face).

Taxon and name	Cultured algal strain	Mycobiont species	Herbarium number	Photobiont acc. 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, CAUP CRYO15	*Diploschistes muscorum* (Scop.) R. Sant.	Peksa 498	**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, CAUP CRYO1	*Stereocaulon pileatum* Ach.	Peksa 999	**AM905999**	**AM906028**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	-	*Cladonia arbuscula* (Wallr.) Flot.	Peksa 789	**AM906000**	**AM906029**
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	-	*Stereocaulon botryosum* Ach.	Talbot 153	DQ229880	DQ229889
Asterochloris irregularis (Hildreth et Ahmadjian) Škaloud et Peksa	-	*Stereocaulon subcoralloides* (Nyl.) Nyl.	Talbot 167	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, CAUP CRYO16	*Lepraria neglecta* (Nyl.) Erichsen	Bayerová 3606	AM900491	**AM906043**

Asterochloris phycobiontica Tschermak-Woess	LEP 7, CAUP CRYO2	*Lepraria neglecta* (Nyl.) Erichsen	Bayerová 3600	**AM906013**	**AM906044**
Asterochloris sp. (clade 6)	LEP 16, CAUP H 1010	*Lepraria neglecta* (Nyl.) Erichsen	Peksa 183	**AM906002**	**AM906031**
Asterochloris sp. (clade 6)	LEP 23, CAUP CRYO3	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 173	**AM906003**	**AM906032**
Asterochloris sp. (clade 6)	LEP 25, CAUP CRYO4	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 204	**AM906004**	**AM906033**
Asterochloris sp. (clade 6)	LEP 30, CAUP CRYO5	*Lepraria neglecta* (Nyl.) Erichsen	Peksa 207	**AM906005**	**AM906034**
Asterochloris sp. (clade 7)	LEP 6, CAUP H 1013	*Lepraria rigidula* (de Lesd.) Tønsberg	Peksa 236	**AM905997**	**AM906023**
Asterochloris sp. (clade 7)	-	*Lepraria rigidula* (de Lesd.) Tønsberg	Peksa 877	**FM955668**	**FM955672**
Asterochloris sp. (clade 7)	-	*Lepraria rigidula* (de Lesd.) Tønsberg	Peksa 900	**FM955669**	**FM955673**
Asterochloris sp. (clade 8)	-	*Cladonia fimbriata* (L.) Fr.	Peksa 796	**FM945358**	**FM955674**
Asterochloris sp. (clade 8)	-	*Cladonia rei* Schaer.	Peksa 787	**FM945380**	**FM955675**
Asterochloris sp. (clade 9)	-	*Pilophorus* cf. *cereolus* (Ach.) Th. Fr.	Nelsen 2233f	DQ229883	DQ229895
Asterochloris sp. (clade 9)	-	*Stereocaulon* sp.	Nelsen 2181b	DQ229884	DQ229896
Asterochloris sp. (clade 10)	LEP 10, CAUP H 1009	*Lepraria borealis* Loht. et Tønsberg	Bayerová 3401	AM900492	**AM906045**
Asterochloris sp. (clade 10)	LEP 15, CAUP CRYO6	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 231	**AM906014**	**AM906047**
Asterochloris sp. (clade 10)	LEP 34, CAUP CRYO7	*Lepraria borealis* Loht. et Tønsberg	Bayerová 3402	**AM906015**	**AM906048**
Asterochloris sp. (clade 10)	LEP 36, CAUP CRYO8	*Lepraria nylanderiana* Kümmerl. & Leuckert	Peksa 542	AM900493	**AM906046**
Asterochloris sp. (clade 10)	CLAD 1, CAUP CRYO9	*Cladonia foliacea* (Huds.) Willd.	Peksa 1008	**AM906016**	**AM906049**
Asterochloris sp. (clade 11)	LEP 5, CAUP H 1011	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 235	**AM905995**	**AM906021**
Asterochloris sp. (clade 11)	LEP 4, CAUP CRYO17	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 234	**AM905994**	**AM906020**

Asterochloris sp. (clade 11)	LEP 33, CAUP CRYO18	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 225	**AM905996**	**AM906022**
Asterochloris sp. (clade 11)	-	*Lepraria lobificans* Nyl.	Nelsen 3974, 154	DQ229877	DQ229898
Asterochloris sp. (clade 11)	-	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Nelsen 3966, L36	EU008664	EU008697
Asterochloris sp. (clade 11)	-	*Lepraria lobificans* Nyl.	Nelsen 3960, L12	EU008675	EU008704
Asterochloris sp. (clade 11)	-	*Lepraria lobificans* Nyl.	Nelsen 3973, 153	EU008678	EU008707
Asterochloris sp. (clade 11)	-	*Lepraria* sp.	Nelsen 2453, L59	EU008691	EU008716
Asterochloris sp. (clade 12)	-	*Cladonia fimbriata* (L.) Fr.	Peksa 815	**FM945359**	**FM955676**
Asterochloris sp. (clade 12)	-	*Cladonia rei* Schaer.	Peksa 921	**FM945378**	**FM955677**
Asterochloris sp. (clade 12)	-	*Stereocaulon paschale* (L.) Hoffm.	Talbot 101	DQ229887	DQ229891
Asterochloris sp. (clade 14)	LEP 31, CAUP H1012	*Lepraria rigidula* (de Lesd.) Tønsberg	Peksa 186	**AM905992**	**AM906017**
Asterochloris sp. (clade 14)	LEP 32, CAUP CRYO10	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 185	**FM955666**	**FM955670**
Asterochloris sp. (clade 14)	LEP 55, CAUP CRYO19	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 551	**FM955667**	**FM955671**
Asterochloris sp. (clade 16)	LEP 13, CAUP H 1014	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 166	**AM906008**	**AM906037**
Asterochloris sp. (clade 16)	LEP 1, CAUP CRYO11	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 233	**AM906006**	**AM906035**
Asterochloris sp. (clade 16)	LEP 2, CAUP CRYO12	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 196	**AM906007**	**AM906036**
Asterochloris sp. (clade 16)	LEP 27, CAUP CRYO13	*Lepraria caesioalba* (de Lesd.) J.R.Laundon	Peksa 194	**AM906009**	**AM906038**
Asterochloris sp. (clade 16)	LEP 28, CAUP CRYO14	*Lepraria alpina* (de Lesd.) Tretiach et Baruffo	Peksa 192	**AM906010**	**AM906039**
Asterochloris sp. (clade 16)	DIP 1, CAUP CRYO20	*Diploschistes muscorum* (Scop.) R. Sant.	Peksa 182	**AM906011**	**AM906040**
Asterochloris sp. (clade 16)	-	*Cladonia cf. bacillaris* (Ach.) Nyl.	Nelsen 3950	DQ229878	DQ229892

Asterochloris sp.	-	*Lepraria* sp.	Nelsen 2166a, L18	EU008687	EU008714
Asterochloris sp.	-	*Lepraria* sp.	Nelsen 2211a, L54	EU008684	EU008711
Asterochloris sp.	-	*Lepraria* sp.	Nelsen 2585, L60	EU008690	EU008715
Asterochloris sp.	-	*Lepraria nigrocincta* Diederich, Sérus. et Aptroot	Nelsen 3637b, L55	EU008681	EU008710
Asterochloris sp.	-	*Stereocaulon vesuvianum* Pers.	Talbot 281	DQ229885	DQ229888
Asterochloris sp.	-	*Stereocaulon tomentosum* Th. Fr.	Talbot 400	DQ229882	DQ229893
Asterochloris sp.	-	*Stereocaulon saxatile* H. Magn.	Talbot KIS 187	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. 4).

	#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	21, 27
Calicium	Physciaceae, Teloschistales, Lecanoromycetes	1	22
Cetraria	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Cladia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	18, 21, 25, 26
Cladonia	Cladoniaceae, Lecanorales, Lecanoromycetes	69	1, 2, 3, 4, 7, 8, 9, 11, 14, 15, 16, 17, 19, 20, 21, 28, 29, 30
Clauzadea	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	2
Cyphelium	Physciaceae, Teloschistales, Lecanoromycetes	1	22
Diploschistes	Thelotremataceae, Ostropales, Lecanoromycetes	2	11, 13, 30
Evernia	Parmeliaceae, Lecanorales, Lecanoromycetes	2	16
Hypogymnia	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Hypotrachyna	Parmeliaceae, Lecanorales, Lecanoromycetes	1	12
Chaenotheca	Coniocybaceae inc. sed., Ascomycota	1	22
Lecidea s.l.	Lecideaceae, Lecanoromycetidae inc. sed., Lecanoromycetes	1	23
Lepraria	Stereocaulaceae, Lecanorales, Lecanoromycetes	12	2, 5, 10, 17, 18, 19, 24, 30
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	23
Pycnothelia	Cladoniaceae, Lecanorales, Lecanoromycetes	1	21
Squamarina	Stereocaulaceae, Lecanorales, Lecanoromycetes	2	6, 23
Stereocaulon	Stereocaulaceae, Lecanorales, Lecanoromycetes	8	1, 3, 15, 17, 21, 30
Usnea	Parmeliaceae, Lecanorales, Lecanoromycetes	3	16
Verrucaria	Verrucariaceae, Verrucariales, Chaetothyriomycetes	1	8
Xanthoria	Teloschistaceae, Teloschistales, Lecanoromycetes	1	3

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