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# ASSEMBLING THE CHALLENGING PUZZLE OF ALGAL BIODIVERSITY: SPECIES DELIMITATION WITHIN THE GENUS *ASTEROCHLORIS* (TREBOUXIOPHYCEAE, CHLOROPHYTA)[1]

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**The genus *Asterochloris* represents one of the most common, widespread, and diverse taxa of lichen photobionts. In this report, we describe and characterize six new species (*A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprarii*, *A. lobophora*, and *A. woessiae*) that were identified during our recent investigation of photobiont diversity. We found that the species differed genetically, morphologically, ecologically, and with respect to their mycobiont partners. Statistical analyses revealed significant morphological differentiation of all six newly described species, as well as their separation from previously described *Asterochloris* species. Chloroplast morphology represented the best morphological marker for species delineation. In fact, each species can be recognized by the dominance and unique assemblage of particular chloroplast types. Although genetically well recognized by rapidly evolving internal transcribed spacer rDNA and actin intron markers, all 13 investigated *Asterochloris* species shared identical small subunit rDNA sequences. We therefore demonstrated that morphologically and ecologically diverse species can frequently be grouped into a single taxonomic unit in whole-transcriptome sequencing studies, considerably affecting the resulting estimates of species diversity. Finally, we demonstrated the presence of isogamous sexual reproduction in *Asterochloris*, disputing the current symbiotic dogma of the loss of sexual reproduction in algal symbionts.**

***Key index words:* *Asterochloris*; cryptic species; green algae; lichens; morphology; phylogeny; speciation; symbiosis; taxonomy**

***Abbreviations*: BI, Bayesian inference; BIC, Bayesian information criterion; CAUP, Culture Collection of Algae of the Charles University in Prague, Czech Republic; CBCs, compensatory base changes; CM, confocal microscopy; GDA, General discriminant analysis; ITS, internal transcribed spacer; MCMC, Markov chain Monte Carlo; ML, maximum likelihood; NGS, next-generation sequencing; OTUs, operational taxonomic units; PCA, principal component analysis; rbcL, ribulose-bisphosphate carboxylase; SAG, Culture Collection of Algae at the University of Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, USA; wMP, weighted maximum parsimony**

Species are fundamental units of biology, comparable to atoms in chemistry or theorems in mathematics. Therefore, the proper delimitation of species is essential for both biologists and the general public. Species delimitation is a fundamental requirement for our understanding of ecosystems and biodiversity, which is necessary for effective decision making about conservation efforts. In addition, taxonomy is a language used by scientists to help the public recognize the diversity, ecology, distribution, and evolutionary history of living organisms. However, evolving over time, species are not unchanging entities. We are therefore surrounded by a plethora of species that vary based on their evolutionary ages, which can make it extremely difficult to perform species identification and delimitation. In fact, the issue of species delimitation has been further complicated by the species problem, that is, the difficulty in defining the concept of species. To date, a wide range of species concepts have been proposed, many of which are associated with several definitions. Moreover, many of these concepts are incompatible in that they can lead to different conclusions concerning the boundaries and number of species (De Queiroz 2007).

In protists, the problem of species delimitation is enhanced by the near absence of morphological features that could be used to clearly distinguish one species from another. As a consequence, a high level of hidden diversity is usually present within nominal, morphologically defined species and genera. Hidden, morphologically highly similar species are frequently described in green algae (Lewis and Flechtner 2004, Fawley et al. 2011, Demchenko

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et al. 2012), chrysophytes (Škaloud et al. 2012, 2014, Jo et al. 2013), diatoms (Mann et al. 2004, Lundholm et al. 2012), ciliates (Quintela-Alonso et al. 2013), and heterotrophic flagellates (Hausmann et al. 2006, Harper et al. 2009). Particularly in green algae, authors often refrain to differentiate the hidden species morphologically, and they define these species according to the phylogenetic species concept (Bock et al. 2011, Krienitz et al. 2011, 2012, Fučíková et al. 2012).

In this study, we focused on species delimitation within the genus *Asterochloris* Tschermak-Woess, one of the most common lichen photobionts. This genus was described by Tschermak-Woess (1980), who differentiated it from the closely related *Trebouxia* Puymaly based on chloroplast morphology. Subsequently, molecular investigations revealed the paraphyly of the genus *Trebouxia* (Friedl and Zeltner 1994, Friedl and Rokitta 1997) and the close relationship of several *Trebouxia* species with the genus *Asterochloris* (Helms et al. 2001, Piercey-Normore and DePriest 2001, Škaloud and Peksa 2008). Splitting of the genus *Trebouxia*, as well as formal delineation of the genus *Asterochloris*, was proposed by Škaloud and Peksa (2010), who also emphasized a huge amount of hidden diversity within the latter genus. According to current knowledge, the genus *Asterochloris* represents one of the most common lichen symbionts, occurring in thalli of more than 20 lichen genera worldwide (Piercey-Normore and DePriest 2001, Yahr et al. 2004, 2006, Cordeiro et al. 2005, Nelsen and Gargas 2006, 2008, Beiggi and Piercey-Normore 2007, Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

During our recent investigation of *Asterochloris* photobionts, we identified a number of new lineages occurring in lichen thalli sampled across Europe (Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011). Apart from their delimitation by unique internal transcribed spacer (ITS) rDNA and actin I sequences, the lineages were significantly differentiated by their substrate and climatic preferences (Peksa and Škaloud 2011), suggesting that, in fact, they represent hidden species. However, the virtual identity of all three published *Asterochloris* SSU rDNA sequences (i.e., *A. erici* —AB080310, *A. magna*—Z21552, and *A. phycobiontica* —GU017647) could call determining these lineages as hidden species into question.

The principal aim of this study was to assess whether genetically differentiated *Asterochloris* lineages could be considered to represent distinct, well-defined species. In particular, we aimed to morphologically differentiate among the 13 lineages, selected to include cultured photobiont strains obtained either from personal or public culture collections. Apart from the seven currently accepted *Asterochloris* species, we investigated six additional lineages representing the putative new species. We performed a detailed morphological investigation of all photobiont strains and investigated whether the lineages could be morphologically delineated. Since species of *Trebouxia* and *Asterochloris* are traditionally differentiated based on their chloroplast morphology, we investigated the chloroplast structure and development of cultivated photobiont cells using modern light and confocal microscopy (CM). Finally, we took advantage of previously published diversity surveys to trace the genetic diversity, ecology, biogeography, and mycobiont specificity of each lineage.

## MATERIALS AND METHODS

*Origin and cultivation of investigated strains.* The majority of strains used in this study were isolated by Škaloud and Peksa (2010) from the lichen thalli sampled in central and eastern Europe (Table S1 in the Supporting Information). The remaining strains were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX), the Culture Collection of Algae at the University of Göttingen, Germany—(SAG), and the Culture Collection of Algae and Protozoa at Argyll, United Kingdom—CCAP. The strains were grown on 2% agarized Bold's basal medium as modified by Bischoff and Bold (1963). All cultures were maintained at a temperature of 15°C, under constant illumination of 7–15 μmol photons $\cdot$ $m^{-2}$ $\cdot$ $s^{-1}$ (cooling box Helkama C5G).

*Morphological observations and statistical analyses.* To obtain a detailed morphological characterization of particular *Asterochloris* lineages, we investigated the cultivated strains by both conventional light and CM. Light microscopy observations were performed using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a differential interference contrast. For CM, a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an argon–krypton laser was used. We applied a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. A series of optical sections through chloroplasts were captured and used for 3-dimensional reconstruction of their morphology. The chloroplast reconstructions were produced by the ImageJ 1.34p program (Abramoff et al. 2004), using the "Volume viewer" plugin.

Individual strains were regularly observed during the 3-month period of culturing, to well characterize the overall morphological variability. Zoospore formation was induced by transferring the cultures to a 1% glucose solution (Hildreth and Ahmadjian 1981). Pyrenoid was visualized by staining with a chloriodine solution (an aqueous solution of 5 g I2 and 10 g of 2,2,2-trichlor-1,1-ethandiol in 5 mL of distilled water). Since some lineages were represented only by a single or two cultured strains, we repeated the morphological investigation of selected strains in a half-year interval, after the inoculation of cells onto the fresh agar plates. During each investigation, the following characters were observed: (i) the average cell width (calculated from at least 45 replicates); (ii) cell shape (a portion of spherical, oval, and pyriform cells); (iii) the maximum number of pyrenoids per cell; (iv) the number of aplanospores per sporangia (16, 32, 48, or 128 spores); (v) chloroplast shape (a portion of following chloroplast types as viewed in CM: shallowly lobed, deeply lobed, crenulate, parietal, echinate, flat lobed, globular); and (vi) chloroplast lobe termination (a portion of following lobe termination types as viewed in CM: elongated, simple, flat, finger like, not formed). Statistical analyses of measured data (principal component and general discriminant analyses)

were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). All graphs were created in R (R Core Team 2014), using the package ggplot2 (Wickham 2009).

*DNA extraction, PCR, and sequencing.* To well characterize the particular *Asterochloris* lineages, we investigated the genetic variation at four loci including the slowly evolving SSU rRNA and rbcL genes, and the rapidly evolving ITS rDNA and actin type I intron markers. Most of the analyzed ITS rDNA and actin sequences originated from our previous studies (Škaloud and Peksa 2010, Peksa and Škaloud 2011). However, to gain a better resolution of species relationships, we additionally obtained 12 ITS rDNA and 7 actin sequences from lichen thalli and cultured *Asterochloris* strains, respectively (Table S1).

Total genomic DNA was isolated following the standard cetyl trimethylammonium bromide protocol (Doyle and Doyle 1987). The amplification of SSU rRNA and *rbcL* genes was performed as described in Neustupa et al. (2013), using the primers 18S-F (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 18S-R (5′-TGA TCC TTC TGC AGG TTC ACC TAC G-3′; Katana et al. 2001), and primers PRASF1 (5′-ATG GTT CCA CAA ACA GAA AC-3′) and PRASR1 (5′-TTG TCA ATA GTA TCA AAT TC-3′; Sherwood et al. 2000). The amplification of ITS rDNA and actin type I locus was performed as described in Peksa and Škaloud (2011), using the primers nr-SSU-1780 (5′-CTG CGG AAG GAT CAT TGA TTC-3′; Piercey-Normore and DePriest 2001) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al. 1990), and primers ActinF2 Astero (5′-AGC GCG GGT ACA GCT TCA C-3′) and ActinR2 Astero (5′-CAG CAC TTC AGG GCA GCG GAA-3′; Škaloud and Peksa 2010). The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and purified using the JetQuick PCR Purification kit (Genomed). The purified amplification products were sequenced using the PCR primers with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730XL) in Macrogen Corp. (Seoul, Korea).

*Sequence analyses.* The newly determined sequences were aligned to other sequences from the GenBank database. Three different alignments were constructed for the phylogenetic analyses: (i) an SSU rDNA alignment of 22/38 unique/total sequences of Trebouxiales, (ii) an *rbcL* alignment of 34/39 unique/total sequences of Trebouxiales, and a (iii) concatenated ITS rDNA + actin alignment of 63/79 unique/total *Asterochloris* sequences selected to encompass all known lineages characterized by both loci. The sequences were aligned using MAFFT v. 6 software (Katoh et al. 2002) under the Q-INS-I strategy and checked for obvious sequencing errors. The alignment of actin sequences was improved by eliminating the ambiguously aligned regions using the program Gblocks v. 0.91b (Castresana 2000). The resulting alignments had lengths of 1772 (SSU rDNA), 1158 (*rbcL*), and 1142 (ITS rDNA + actin) characters, respectively. All alignments were submitted to TreeBase (http://www.treebase.org/treebase-web/home.html) and are available under No. S16886.

For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba et al. 2012). This BIC-based model selection procedure selected the following models: (i) TrNef + I + Γ for SSU rDNA, (ii) TIM2 + Γ for the first codon position of the *rbcL* gene, (iii) JC + I for the second codon position of the *rbcL* gene, (iv) TIM3 + I for the third codon position of the *rbcL* gene, (v) TrNef + Γ for ITS1, (vi) K80 + Γ for ITS2, actin exon, and actin intron 248, (vii) JC for 5.8S rDNA, and (viii) HKY + Γ for actin intron 206.

The phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist et al. 2012). With the exception of SSU rDNA data, the analyses were carried out on partitioned data sets using the different substitution models selected by jModelTest 2.1.4. For those models having complicated substitution types, a mixed substitution type was selected to sample across the substitution model space in the Bayesian Markov chain Monte Carlo (MCMC) analysis itself. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for five million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF values of SSU rDNA, *rbcL*, and concatenated ITS rDNA + actin analyses were 0.0008, 0.0035, and 0.0053, respectively. Finally, the burn-in values were determined using the "sump" command.

Bootstrap analyses were performed by maximum-likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl 2006), and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudoreplicates) using automatic termination (genthreshfortopoterm command set to 100,000). The analyses were performed on partitioned data sets using the different substitution models selected by jModelTest 2.1.4. The wMP bootstrapping (1,000 pseudoreplicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1,000. New weights were based on the mean fit values for each character over all trees in the memory.

To show the genetic diversity within the newly characterized lineages, we constructed the haplotype networks on the basis of maximum parsimony analyses of all available sequences, using the Haplotype Viewer (G. Ewing, available at www.cibiv.at/~greg/haploviewer).

## RESULTS

*Analyses of molecular data.* BI of the SSU rDNA and *rbcL* data yielded similar tree topologies, resolving *Asterochloris*, *Trebouxia*, and *Myrmecia* Printz as well-defined, distinct genera (Fig. 1). In the SSU rDNA analysis, a clade of environmental sequences from soil samples (Lesaulnier et al. 2008) was additionally inferred in the affiliation of the genus *Asterochloris*. Comparison with other SSU rDNA sequences showed that six investigated *Asterochloris* strains (SAG 26.81, UTEX 911, Bayerová 3401, Peksa 183, Peksa 236, and Peksa 999) contained IB3 group I introns at position 516 relative to the *Escherichia coli* coding region. The exon SSU rDNA sequences of all investigated *Asterochloris* strains were completely identical. Resequencing of a single genetically distinct strain (*A. magna* UTEX 902, accession Z21552) confirmed that all nucleotide differences correspond to sequencing errors (Fig. 1a). The *Asterochloris rbcL* sequences were slightly different from each other, but analysis of these sequences did not reveal any highly supported clades with the exception of a lineage comprising *A. glomerata* and *A. irregularis* strains (Fig. 1b).

Bayesian analysis of the concatenated ITS rDNA and actin data set revealed the existence of more

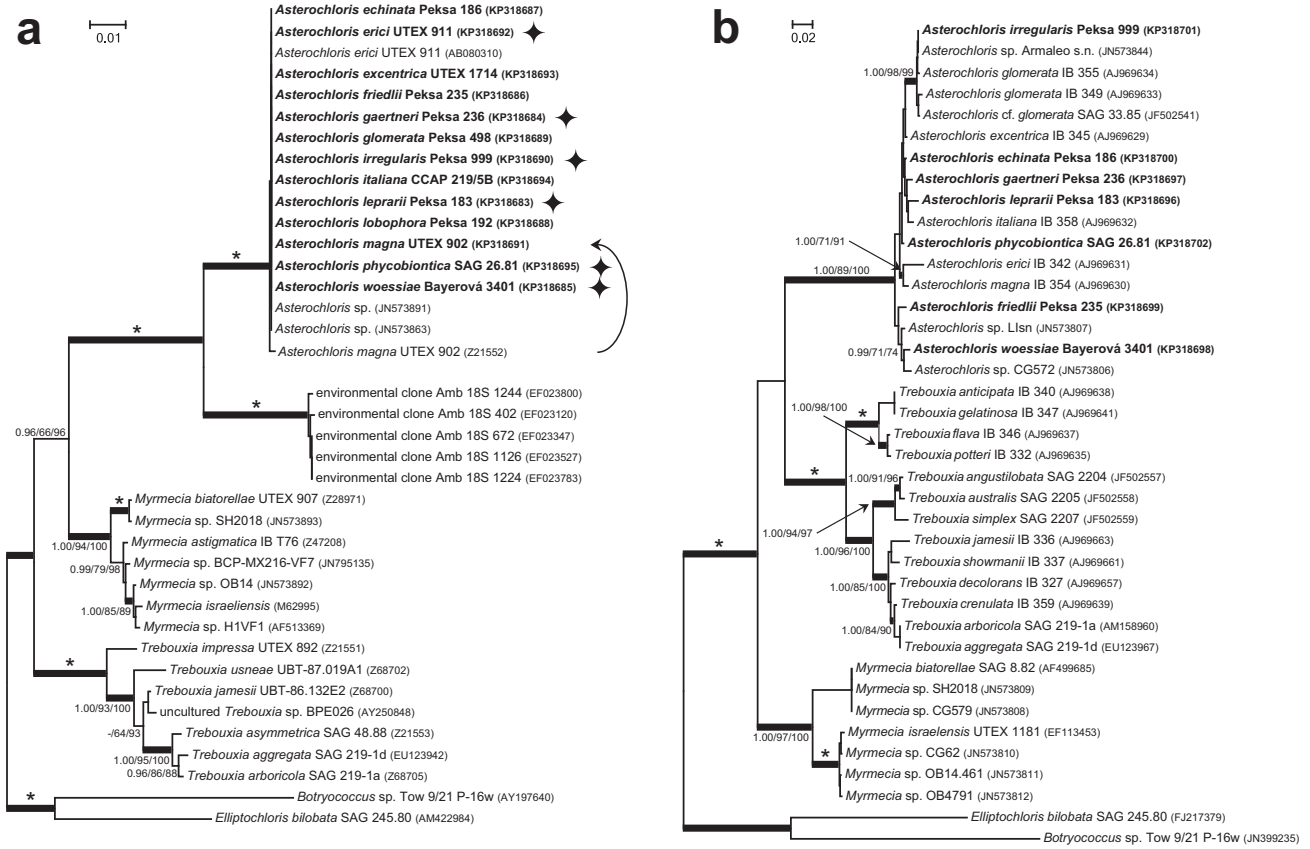


FIG. 1. Phylogeny of the Trebouxiales obtained by Bayesian inference of the SSU rDNA (a) and *rbc*L (b) data sets. Values at the nodes indicate statistical support estimated by three methods: MrBayes posterior-node probability (left), maximum-likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Full statistical support (1.00/100/100) is marked with an asterisk. Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly sequenced strains are marked in bold. Those sequences containing the IB3 group I introns are marked by stars. An arrow indicated the corrected phylogenetic position of the strain UTEX 902 (*Asterochloris magna*), which SSU rDNA sequence was deposited in GenBank with putative sequencing errors. Scale bar represents the expected number of substitutions per site.

than 20 well-resolved lineages within the genus *Asterochloris* (Fig. 2). The relationships among the lineages correspond well with the phylogeny presented by Škaloud and Peksa (2010), including the presence of three moderately to well-supported major clades, A, B, and C. All seven formerly described species (*A. erici*, *A. excentrica*, *A. glomerata*, *A. irregularis*, *A. italiana*, *A. magna*, and *A. phycobiontica*) formed well-recognized, distinct lineages. The *Asterochloris* cultures that we isolated during our recent investigation of lichen photobionts clustered within the six distinct lineages recognized by Škaloud and Peksa (2010) as lineages 6, 7, 10, 11, 14, and 16. Lineages 6 and 7 (here referred to as *A. leprarii* sp. nov. and *A. gaertneri* sp. nov.) were inferred to belong within clade B, together with *A. excentrica* and three additional lineages. The remaining four lineages (here referred to as *A. echinata* sp. nov., *A. friedlii* sp. nov., *A. lobophora* sp. nov., and *A. woessiae* sp. nov.) were inferred to be members of clade C, including *A. italiana*, *A. phycobiontica*, and five additional lineages. Within clade C, the relationship among the lineages remained unresolved, with the exception of the close, significant relationship between *A. phycobiontica* and *A. lobophora*.

The comparison of all available ITS rDNA sequences pointed to wide differences in genetic variability within the six newly recognized species (Fig. 2b). *A. leprarii* and *A. gaertneri* showed almost no intraspecific genetic diversity. Of the 30 investigated *A. gaertneri* isolates, only one differed by a single nucleotide substitution. On the other hand, *A. lobophora* represented the most diverse species, containing a total of 18 different ITS rDNA genotypes. *A. lobophora* also stands out due to its higher occurrence in *Cladonia* lichens.

*Morphological analyses.* The diameter of vegetative cells varied, ranging from 4 to 29 μm. The cells were generally spherical and occasionally oval or pyriform. The cell wall was thin, and occasionally, a flat, localized thickening of the wall was detected in mature cells. In old cultures, the walls of some cells were slightly thickened along their entire surface. A single nucleus with distinct nucleolus was situated parietally in the broad chloroplast infolding.

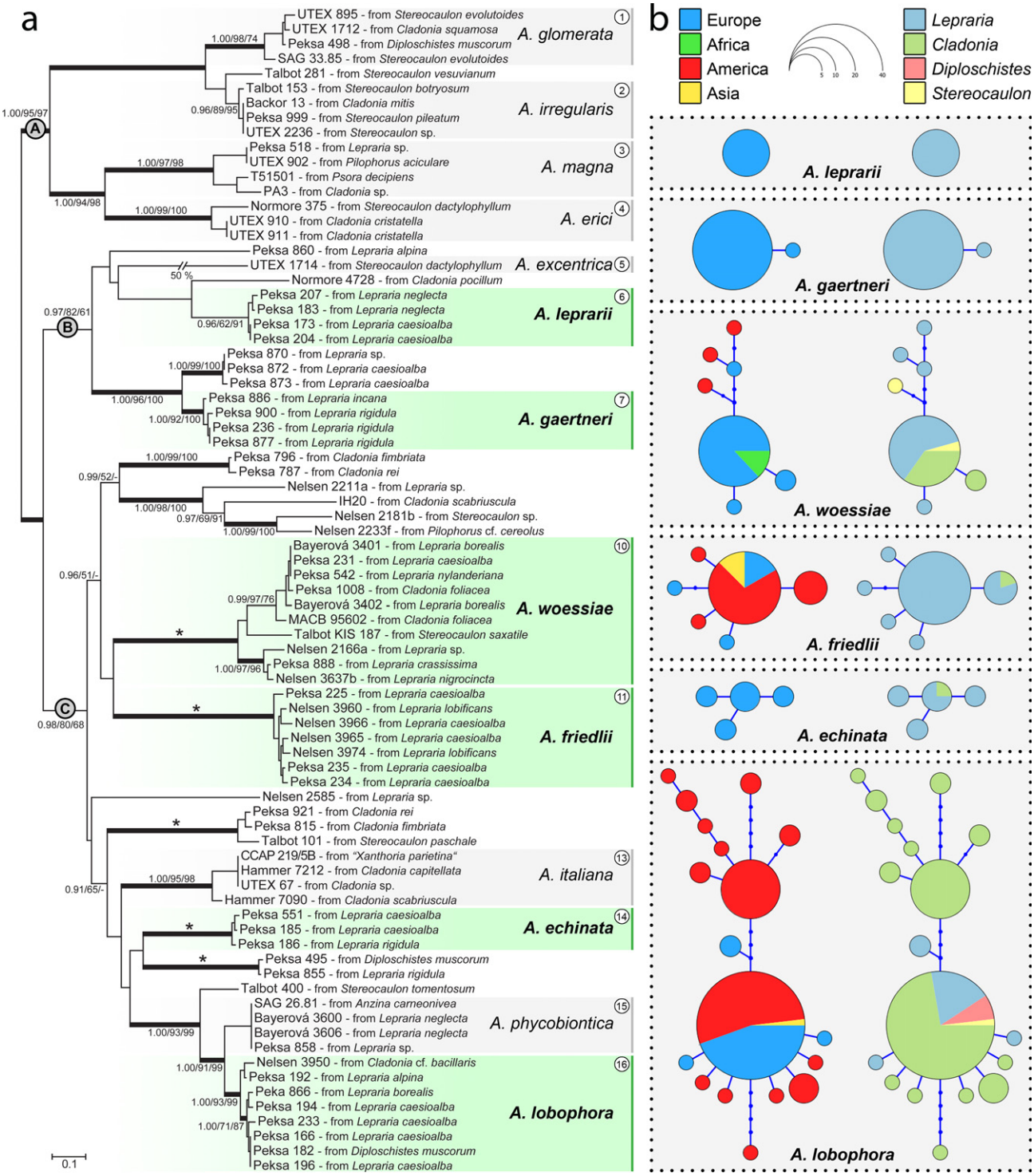


FIG. 2. Genetic diversity within the genus *Asterochloris*. (a) The Bayesian majority rule tree based on the concatenated ITS rDNA + actin alignment. See Figure 1 for the explanation of node values. Clade numbering and affiliation into the three major clades (A-C) follows Škaloud and Peksa (2010). Scale bar represents the expected number of substitutions per site. (b) Statistical parsimony haplotype networks of all available ITS rDNA sequences, showing the intraspecific diversity within the newly proposed species. Genotypes are colored according to the sampling continent (on the left) and respective mycobiont symbiotic partner (on the right). The sizes of circles representing genotypes reflect the number of sequences that share a genotype. Inferred intermediate haplotypes that were either not sampled or are extinct are represented by small noncolored circles.

The majority of the cell volume was occupied by the chloroplast. In young cells, the chloroplast was parietal or ribbon shaped. Soon, it shifted to a central position and began to develop into a massive, lobed form. Mature vegetative cells therefore contained a central axial chloroplast with variously arranged lobes reaching the cell periphery. In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transformed into the parietal type, with smooth, never lobed margins. After a short time, it began to divide into numerous parts in preparation for asexual reproduction. Taking advantage of laser scanning CM, we recognized seven specific chloroplast types occurring in mature *Asterochloris* cells (Fig. 3), as follows: (i) a *deeply lobed* type, characterized by long, branched, or unbranched lobes emerging directly from the thin chloroplast layer spreading around the pyrenoid ("Tieflappig Typ" sensu, Gärtner 1985a); (ii) a *shallowly lobed* type, which is similar to the previous type but differs in that the chloroplast lobes emerge from the central mass of the chloroplast layer encircling the pyrenoid ("Normaltyp" sensu, Gärtner 1985a); (iii) a *crenulate* type, distinguished by a central, massive chloroplast with a regularly nodulated surface ("Crenulater Typ" sensu, Gärtner 1985b); (iv) a *parietal* type, characterized by parietally positioned nodulated chloroplast with the margins extended into divided finger-like lobes; (v) a *flat lobed* type, representing an axial chloroplast with long lobes that appear flattened over their entire length; (vi) an *echinate* type, characterized by numerous thin radial lobes emerging uniformly from the central mass of the chloroplast layer; and (vii) a *globular* type, a simple spherical chloroplast without, or with very shallow, lobes.

In addition to these seven morphological chloroplast types, we distinguished four types of lobe terminations (Fig. 3), as follows: (i) an *elongated* type, with lobes extending longitudinally at their ends, therefore giving the chloroplast a finny appearance in surface view ("Rippenförmig Typ" sensu, Gärtner

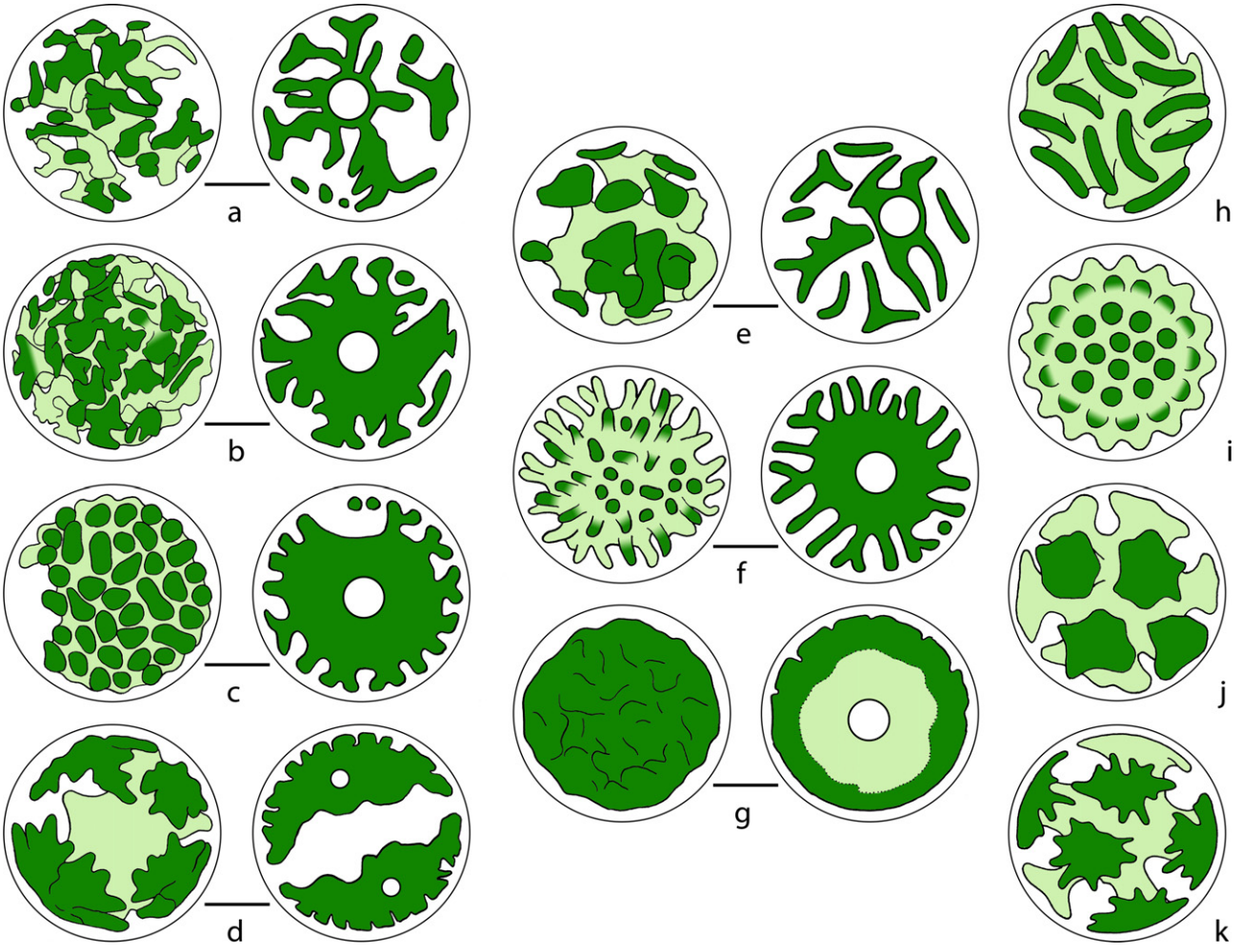


FIG. 3. Schematic drawings of particular chloroplast and lobe termination types in *Asterochloris*. (a–g) Chloroplast types (left: surface view; right: view in optical section): (a) deeply lobed, (b) shallowly lobed, (c) crenulate, (d) parietal, (e) flat lobed, (f) echinate, (g) globular. (h–k) Lobe termination types: (h) elongated, (i) simple, (j) flat, (k) finger-like.

1985a); (ii) a *simple* type, characterized by simply terminated lobes at their ends; (iii) a *flat* type of chloroplast lobe, terminated by irregular plates, perpendicularly oriented with respect to the lobe axis; and (iv) a *finger-like* type, distinguished by lobes branched into several finger-like projections. These projections were perpendicularly oriented with respect to the lobe axis, spreading below the plasma membrane.

Pyrenoids were present in all *Asterochloris* species except *A. magna*. While *A. erici* and *A. lobophora* had single pyrenoids, the cells usually contained one to several pyrenoids lying in the chloroplast center. Often, one large centrally located pyrenoid was surrounded by several smaller satellite pyrenoids, which were created by budding. The pyrenoids were generally distinct; only in *A. erici* did the pyrenoid gradually change over to a chloroplast matrix without a distinct pyrenoid margin. Various structures could occasionally be observed in the pyrenoid matrix by both conventional light and CM. The pyrenoids were granulated, striated, or perforated. In *A. phycobiontica*, the pyrenoid contained distinct rings of puzzling origin. The frequency and markedness of subpyrenoidal structures significantly increased with cell age. Pyrenoids were usually surrounded by a conspicuous starch sheet, which could be visualized by staining with chloriodine solution.

To investigate morphological differences between the 13 recognized *Asterochloris* species in detail, we characterized each species based on a number of features, including cell shape, dimensions, and chloroplast morphology (Table S2 in the Supporting Information). We found no significant differences in average cell dimensions among species; however, some species could be distinguished by the prevailing shape of their cells. The closely related *A. glomerata* and *A. irregularis* frequently produced oval and pyriform cells (Fig. 4a); by contrast, these cells were never observed in *A. echinata*. In addition, all investigated species were obviously heterogeneous in the overall morphological complexity of chloroplast types (Fig. 4b). Two to four chloroplast types were usually observed during cell ontogeny. The shallowly lobed chloroplast was the most common type, occurring in 11 of 13 investigated *Asterochloris* species. Four species (*A. glomerata*, *A. irregularis*, *A. woessiae*, and *A. excentrica*) were characterized by the prevailing occurrence of the deeply lobed chloroplast type, while the flat lobed type was only observed in the first three species. By contrast, deeply lobed chloroplasts were never found in *A. phycobiontica* or *A. lobophora*, which were characterized by the presence of crenulate and finger-like types. Two species, *A. magna* and *A. echinata*, could be easily recognized by their specific chloroplast morphology, the former by the presence of the simplest globular type and the latter by the combination of crenulate and echinate types. Finally, *A. italiana* was exceptional because it produced a single, shallowly lobed chloroplast type.

Morphological differences among the species were also observed in the shape of chloroplast lobe terminations (Fig. 4c). Elongated terminations were most commonly produced, which were observed in all species except *A. magna* and *A. echinata*. These two species could clearly be recognized by the common absence of any lobes (*A. magna*) and by the exclusive formation of single lobe terminations (*A. echinata*). Moreover, the prevalence of finger-like node terminations is characteristic of *A. phycobiontica*.

Principal component analysis (PCA) of the entire data set resulted in a relatively well-defined grouping of investigated strains belonging to particular species (Fig. 4d). For example, *A. erici* and *A. magna* were plotted in two distinct clusters in the upper left corner of the PCA plot. On the other hand, several strains belonging to different species were intermixed with each other (e.g., *A. excentrica* and *A. woessiae*). Interestingly, the closely related species pairs (*A. glomerata–A. irregularis* and *A. phycobiontica–A. lobophora*) were obviously similar based on their morphology. General discriminant analysis (GDA) yielded much better grouping of species into separate clusters (Fig. 4, e and f). *A. magna* formed a strong outlying cluster with a negative value on the second GDA axis (Fig. 4e). A scatter plot based on the first and third GDA axes showed the separation of all 13 *Asterochloris* species into distinct clusters (Fig. 4f). Discriminant analysis (DA) indicated strongly significant differentiation among all investigated species (Wilk's $\lambda < 0.00001$; $P < 0.00001$). Forward stepwise analysis selected the globular chloroplast, parietal chloroplast with finger-like lobes, the number of autospores, and the number of pyrenoids as the best discriminating characters. The first and third GDA axes, which well discriminated all investigated species, were highly correlated with the four factors examined. Whereas the first GDA axis was correlated with parietal and flat chloroplast types with elongated lobes (correlation coefficients 0.34 and 0.38, respectively), the third axis was correlated with deeply lobed chloroplasts with elongated lobes and globular chloroplasts (correlation coefficients 0.49 and 0.41, respectively). The globular chloroplast type was also highly correlated with the second GDA axis (correlation coefficient −0.70). The average correct discrimination of individual strains based on their morphology reached 94.3%; that is, only three investigated strains (one *A. excentrica* and two *A. woessiae* strains) were classified incorrectly by the discriminant model.

*Reproduction.* The life cycle and reproductive processes are schematically delineated in Figure 5. Asexual reproduction occurred by the formation of aplanospores, zoospores, and autospores. Autospore production was relatively rare; it was observed only in some species. Autospore production was initiated by slight cell enlargement and subsequent chloroplast division (Fig. 5b). In general, autospores were formed in relatively small

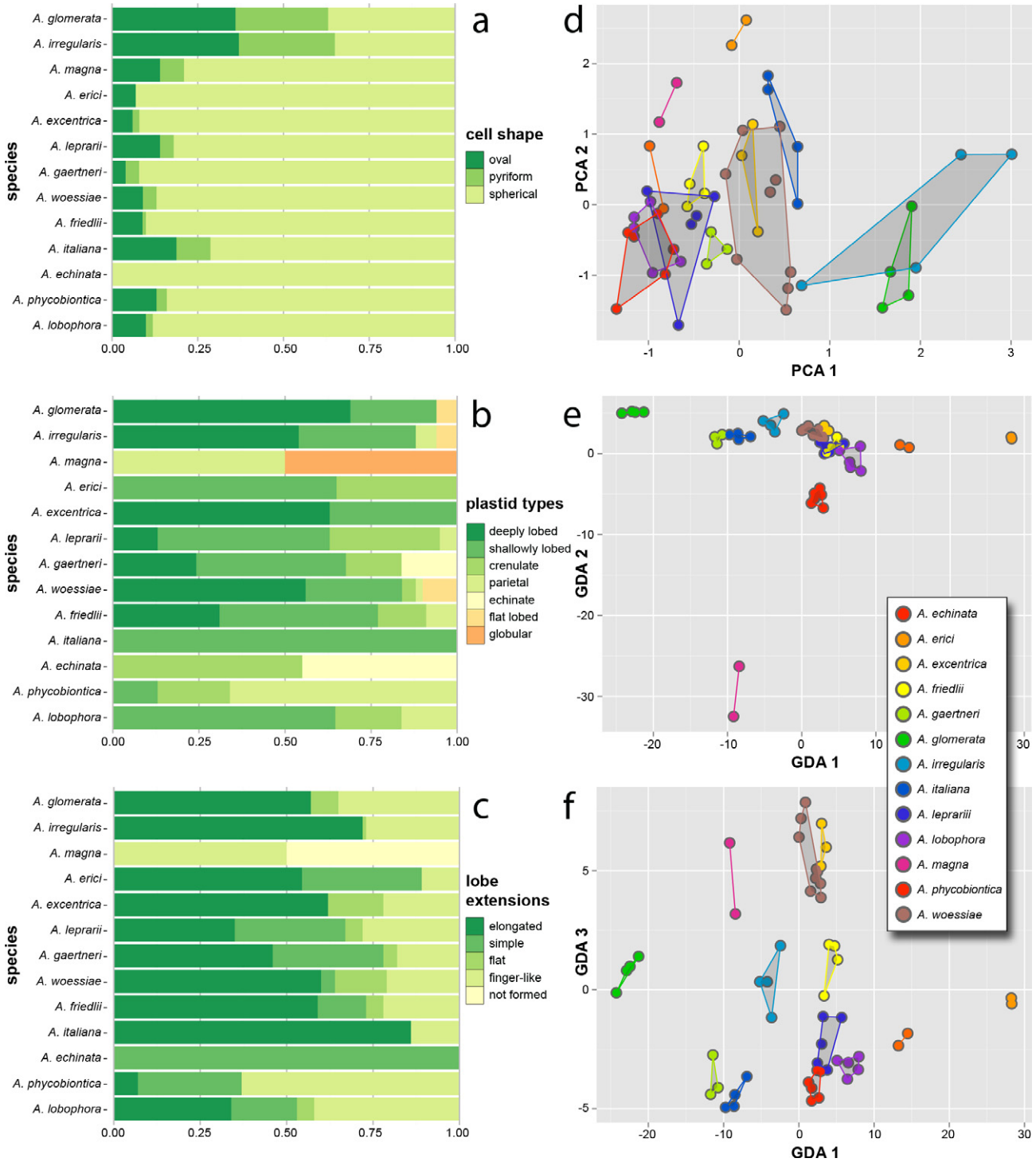


FIG. 4. Morphological comparisons of 13 investigated *Asterochloris* species. (a) Proportion of three different cell shapes. (b) Proportion of seven recognized plastid types. (c) Proportion of five distinguished lobe termination types. (d) Principal component analysis (PCA) of the entire measured morphological features data set. (e, f) General discriminant analysis (GDA) the same data set: (e) the two-dimensional plot of the 1st and 2nd GDA axes, (f) the two-dimensional plot of the 1st and 3rd GDA axes.

numbers (mostly four or eight) and were liberated by either decomposition or rupturing of the mother cell wall, without producing any special openings (Fig. 5d). The formation of aplanospores and zoospores was much more frequent. Prior to the first cleavage, the chloroplast flattened,

migrated to the parietal position, and started to divide (Fig. 5f). Ultimately, a large number of daughter cells (usually 64 or 128) were present within the sporangium (Fig. 5h). Once all the daughter cells formed, a distinction between aplanosporangia and zoosporangia was observable. In the case of aplanospores, the daughter cells rounded and produced a cell wall of their own. Mature aplanospores were liberated by rupturing of the mother cell wall (Fig. 5i). Mature zoosporangia were discernible by the irregular shape of their daughter cells (Fig. 5k). The zoospores were all liberated simultaneously with the rupturing of the mother cell wall, enclosed in a gelatinous vesicle that could slightly evaginate from the mother cell wall during the liberation process. The zoospores were dorsiventrally flattened, with two equal anterior flagella, a posterior chloroplast, a median-to-posterior nucleus, and indistinct stigma. Following liberation from the sporangium, the zoospores swam in a packet, the remaining joined together by their posterior extensions (Fig. 5l). Shortly thereafter, single zoospores started to detach from the packet and swam separately. Despite this, the majority of zoospores still bore posterior extensions even if observed several minutes after separation (Fig. 5n).

Sexual reproduction was very scarce and was observed only twice in *A. woessiae* (strain CAUP H1009). Biflagellate isogamous gametes were morphologically indistinguishable from zoospores. After their fusion, they gave rise to large quadriflagellate

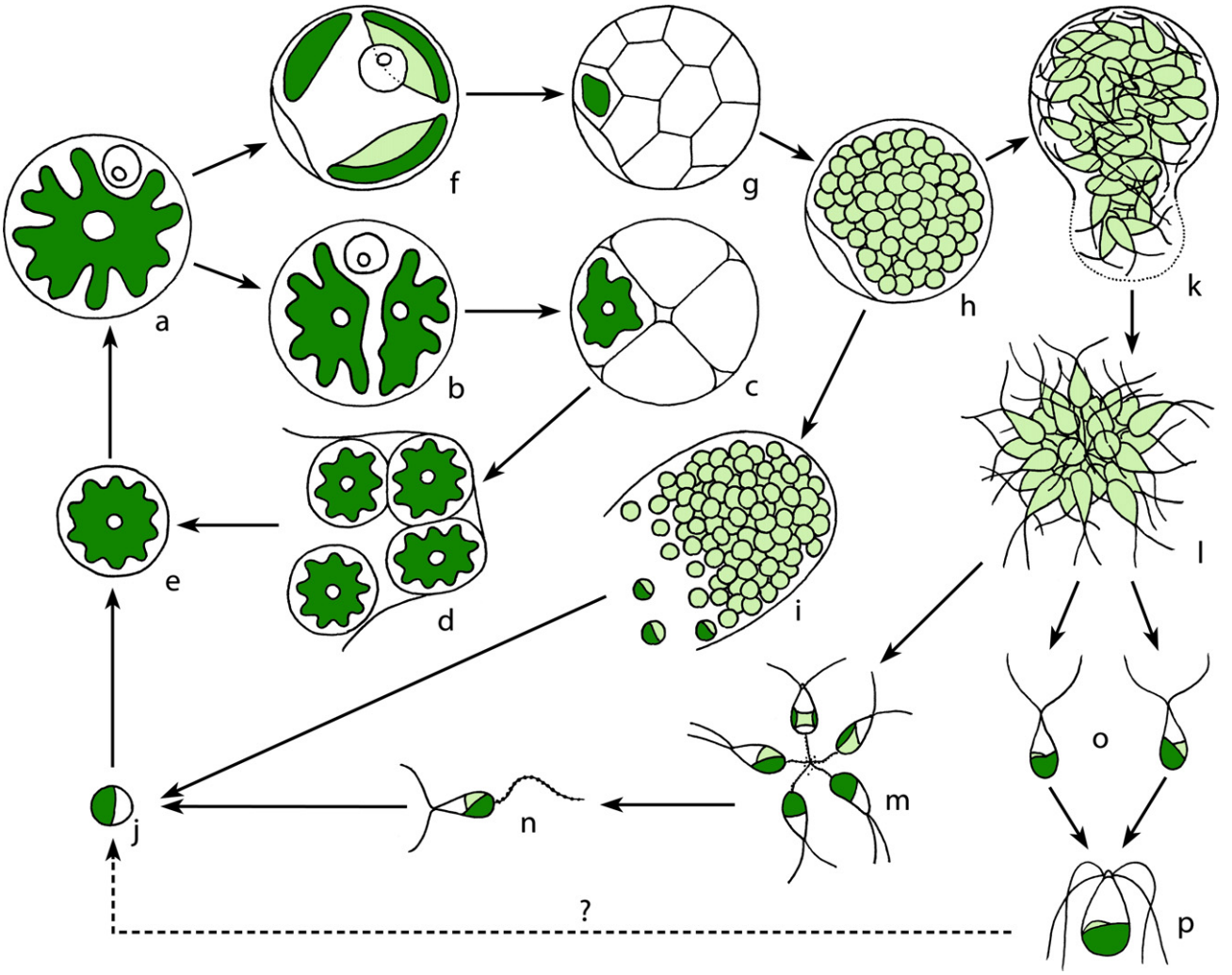


FIG. 5. Schematic representation of the life cycle and reproduction processes in *Asterochloris*. (a) Vegetative cell. (b–e). Autosporogenesis: (b) chloroplast division, (c) young autosporangium, (d) release of autospores, (e) mature autospore. (f–n). Aplano- and zoosporogenesis: (f) chloroplast flattening, note a local thickening of the cell wall, (g) young aplano/zoosporangium, (h) mature aplano/zoosporangium, (i) release of aplanospores, (j) young aplanospore, (k) release of zoospores, note evagination of gelatinous vesicle, (l) zoospore packet, (m) releasing of zoospores from the packet, (n) single zoospore with a posterior extension. (o, p). Sexual reproduction: (o) gametes, (p) planozygote.

planozygotes (Fig. 5p). The germination of planozygotes was not observed.

## TAXA DESCRIPTIONS

In this study, we revealed a clear morphological differentiation of the studied *Asterochloris* lineages. Given the genetic distinctiveness, previously published ecological diversification, and mycobiont specificity of these lineages, we are proposing that they represent new species. Descriptions and characteristics of these new taxa are provided below.

***Asterochloris*** Tschermak-Woess; 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 description*: Cells spherical, occasionally oval or pyriform. Cell wall thin, occasionally with a flat local thickening. Single nucleus situated parietally in the broad chloroplast infolding. Chloroplast single, asteroid, with variously arranged lobes reaching the cell periphery. One to several pyrenoids usually lie in the chloroplast center, surrounded by a conspicuous starch sheet. Prior to aplano- and zoosporogenesis, the chloroplast flattens and assumes a parietal position. Asexual reproduction by usually 64–128 aplanospores and zoospores, occasionally by 2–8 autospores. Zoospores naked, dorsiventrally flattened, with two apical flagella, a posterior chloroplast, a median-to-posterior nucleus, and indistinct stigma. Following liberation from the sporangium, the zoospores shortly swam in a packet, joined together by their posterior extensions. Sexual reproduction scarce, by the fusion of two isogamous gametes. Photobionts of many lichens (genera *Anzina*, *Cladia*, *Cladonia*, *Diploschistes*, *Lepraria*, *Pilophorus*, *Pycnothelia*, *Stereocaulon*, etc.). Widely distributed, cosmopolitan. From the morphologically similar genus *Trebouxia*, it generally differs by the presence of deeply lobed chloroplast, parietal position of chloroplast prior to zoo- or aplanosporogenesis, and the production of aplanospores as a prevailing type of asexual reproduction.

***Asterochloris leprarii*** Škaloud et Peksa **sp. nov.** (Fig. 6, a–k)

Vegetative cells usually spherical, occasionally oval and pyriform, (5-)7.5-24(-28) μm in diameter (Fig. 6, a–c). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells exhibit central chloroplasts of either shallowly lobed (Fig. 6d) or crenulate form (Fig. 6e). Rarely, the deeply lobed (Fig. 6f) and parietal chloroplast (Fig. 6g) is observed as well. The chloroplast lobes can be simply terminated (Fig. 6e), elongated at their ends (Fig. 6f), or finger like (Fig. 6g). Occasionally, the lobe ends are flat (Fig. 6h). The chloroplast contains from one to many pyrenoids. Besides the typical, centrally located pyrenoid, up to seven smaller ones may be present in its vicinity (Fig. 6i). Sometimes, an indistinct granulation or striation can be visible inside pyrenoids. Starch grains are either embedded in a layer around the pyrenoid or distributed evenly throughout the chloroplast. Asexual reproduction by 64–128 aplanospores or 64 zoospores produced in spherical or ellipsoidal sporangia (Fig. 6j). Occasionally, 2–4 autospores are also produced. Zoospores dorsiventrally flattened, drop shaped, arcuate in lateral view, 6–10 μm long and 2.8–4 μm wide, with posterior extensions (Fig. 6k).

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Peksa 183, deposited in the Culture Collection of Algae of the Charles University in Prague (CAUP) as the item TYPE-H 1010.

*Reference strains*: CAUP H 1010, SAG 2280.

*Type locality*: Phycobiont of *Lepraria neglecta*, collected on siliceous rock, Rybárna, Šumava Mts, Czech Republic, May 23, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL (Collection of The West Bohemian Museum in Pilsen), No. 183.

*Etymology*: The species is named in reference to the mycobiont genus *Lepraria* Ach.

*Distribution*: So far known only from Europe: Czech Republic, Norway, Slovakia (Nelsen and Gargas 2008, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

*Ecology*: In temperate Europe, it prefers altitudes of about 800–1,000 m a.s.l; associates with ombrophilic lichens growing on acidic substrates, especially siliceous rocks (Peksa and Škaloud 2011).

*Specificity*: found exclusively in the thalli of lichen genus *Lepraria*.

***Asterochloris gaertneri*** Škaloud et Peksa **sp. nov.** (Fig. 6, l–t)

Vegetative cells spherical, very rarely oval or pyriform, 5.5–26(-29.5) μm in diameter (Fig. 6, k and l). Cell wall thin, seldom a flat local thickening of the cell wall can be observed. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells often possess shallowly lobed axial chloroplasts (Fig. 6, m and n). Deeply lobed (Fig. 6o), crenulate (Fig. 6p), and echinate (Fig. 6, q and r) chloroplast forms observed, as well. The chloroplast lobes are simply terminated, extended longitudinally at their ends, or terminated by finger-like extensions. Flat lobes produced very rarely. The chloroplast contains from one to many distinct pyrenoids. If many, they usually jointly occupy the chloroplast's center (Fig. 6s). Sometimes, an indistinct striation can be visible inside pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 64–128–256 aplanospores or 128 zoospores produced in large spherical or ellipsoidal sporangia

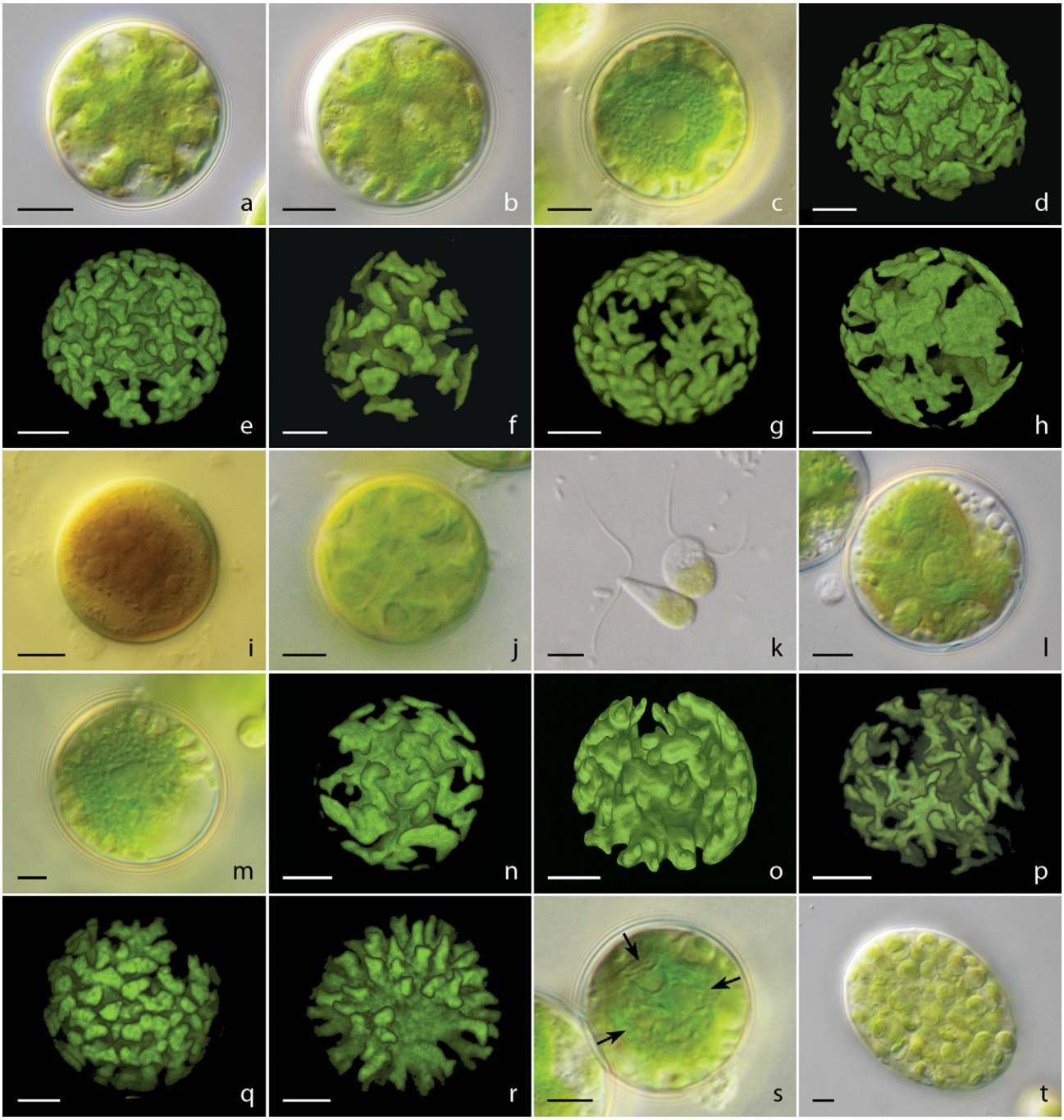


FIG. 6. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris leprarii* and *A. gaertneri*. (a–k) *A. leprarii*. Light micrographs of mature vegetative cells possessing deeply lobed (a), shallowly lobed (b), and crenulate (c) chloroplasts. Confocal reconstructions of shallowly lobed (d), crenulate (e), deeply lobed (f), and parietal (g) chloroplast types. (h) Deeply lobed chloroplast with flat lobe ends. Several pyrenoids occur around the large central pyrenoid (i); cells stained by chloriodine solution. (j) Young aplanosporangium. (k) Zoospores. (l–t) *A. gaertneri*. Light microscopy of shallowly lobed (l) and crenulate (m) chloroplast. Confocal reconstructions of shallowly lobed (n, o), deeply lobed (p), crenulate (q), and echinate (r) chloroplasts. Several pyrenoids of equal size (arrows) occur in the chloroplast's center (s). (t) Mature aplanosporangium; scale bar—5 μm.

(Fig. 6t). Occasionally, 4–8 autospores are also produced. Zoospores dorsiventrally flattened, 6–7.5 μm long and 2.5–4 μm wide.

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Peksa 236, deposited in the CAUP as the item TYPE-H 1013.

*Reference strains*: CAUP H 1013, SAG 2283.

*Type locality*: Phycobiont of *Lepraria rigidula*, collected on bark of *Acer pseudoplatanus*, near Stříbrnická Mt., Králický Sněžník Mts, Czech Republic, February 10, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 236.

*Etymology*: The species epithet is in honor of the work of Dr. Georg Gärtner, who published several reports on *Trebouxia* s.l.

*Distribution*: So far known only from Europe: Czech Republic, Germany, Slovakia (Škaloud and Peksa 2010).

*Ecology*: Associates with lichens growing on tree bark and siliceous rocks, in rain-sheltered situations (ombrophobic); in temperate Europe, it was found at altitudes of about 500–900 m a.s.l (Peksa and Škaloud 2011).

*Specificity*: found exclusively in the thalli of lichen genus *Lepraria*.

***Asterochloris woessiae*** Škaloud et Peksa **sp. nov.** (Fig. 7, a–k)

Vegetative cells usually spherical, rarely oval and pyriform, (5-)6.5–19(-25.5) μm in diameter (Fig. 7a). Cell wall thin, seldom a flat local thickening of the cell wall can be made out (Fig. 7b). Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery (Fig. 7c). Mature cells display a structurally complicated, central, deeply lobed chloroplast with branched lobes that emerge directly from the thin layer spreading around the pyrenoid (Fig. 7d). Mature cell chloroplasts can further exhibit several other ontogenetic stages, alternating during the cell's ontogeny. The chloroplast can be shallowly lobed (Fig. 7c), or appearing flattened over their entire length (Fig. 7e). Rarely, the crenulate (Fig. 7f) and parietal chloroplasts can be formed as well. The chloroplast lobes can be terminated by all four known types, usually by elongated, flat (Fig. 7g), or finger-like extensions. The chloroplast contains 1–3 distinctively delimited pyrenoids (Fig. 7h). Sometimes, an indistinct granulation can be visible inside the pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 128 aplanospores or zoospores produced in large spherical or ellipsoidal sporangia (Fig. 7i). Occasionally, 2–4 autospores are also produced. Zoospores dorsiventrally flattened, 4.5–7.5 μm long and 2.5–4.5 μm wide, with posterior extensions (Fig. 7j). Sexual reproduction by fusion of biflagellate isogamous gametes; planozygotes with four longitudinal flagella (Fig. 7k).

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Bayerová 3401, deposited in the CAUP as the item TYPE-H 1009.

*Reference strains*: CAUP H 1009, SAG 2279.

*Type locality*: Phycobiont of *Lepraria borealis*, collected on sunlit slate rock, Stara planina Mts, Central Balkan National Park, Bulgaria, July 1, 2004. The lichen specimen is deposited in collection of Š. Bayerová-Slavíková in PRA (Herbarium of Institute of Botany of the ASCR, Czech Republic), No. 3401.

*Etymology*: The species epithet is in honor of the work of Dr. Elisabeth Tschermak-Woess, who described the genus *Asterochloris*.

*Distribution*: Cosmopolitan, widely distributed. Europe: Bulgaria, Czech Republic, Great Britain, Slovakia, Spain, Sweden; America: Costa Rica, USA; Africa: Canary Islands (Nelsen and Gargas 2006, Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011, Pino-Bodas et al. 2010).

*Ecology*: Prefers lichens growing on moderately basic substrates (shale, basalt, serpentine rocks etc.) and low altitudes of about 300–600 m a.s.l of temperate Europe (Peksa and Škaloud 2011).

*Specificity*: Found in a number of lichen species belonging to genera *Lepraria*, *Cladonia*, and *Stereocaulon*.

***Asterochloris friedlii*** Škaloud et Peksa **sp. nov.** (Fig. 7, l–t)

Vegetative cells spherical or slightly oval, (4.5-)6–18(-21) μm in diameter (Fig. 7, l and m). Cell wall thin, seldom a flat local thickening of the cell wall can be detected. Chloroplast in young cells assumes the central position with several simple lobes spreading toward the cell's periphery (Fig. 7n). Mature cells generally display a structurally complicated, central, deeply or shallowly lobed chloroplast with branched lobes (Fig. 7, o and p). Sometimes, the crenulate (Fig. 7q) or parietal chloroplast (Fig. 7r) can be formed as well. In mature cells, the chloroplast can be slightly asymmetrically positioned. The chloroplast lobes are mostly extended longitudinally at their ends, but they can be terminated by additional three types, as well. The chloroplast generally contains a single distinct, granulated pyrenoid. Especially in older cells, the pyrenoid buds at its surface and gives rise to several smaller ones in its vicinity (Fig. 7s). Starch grains are embedded in a layer around the pyrenoid in the form of large granules. Asexual reproduction by 64–128 aplanospores or zoospores produced in large spherical, ellipsoidal, or irregular sporangia (Fig. 7t). Zoospores dorsiventrally flattened, 4.5–7 μm long and 3–3.5 μm wide, with posterior extensions.

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Peksa 235, deposited in the CAUP as the item TYPE-H 1011.

*Reference strains*: CAUP H 1011, SAG 2281.

*Type locality*: Phycobiont of *Lepraria caesioalba*, collected on bryophytes on siliceous rock, Klenovský Vepor Mt., Slovenské Rudohorie Mts, Slovakia, July 12, 2004. The lichen thallus is deposited in herbarium of O. Peksa in PL, No. 235.

*Etymology*: The species epithet is in honor of the work of Dr. Thomas Friedl, who published several reports on *Trebouxia* s.l.

*Distribution*: Cosmopolitan, widely distributed. Europe: Czech Republic, Romania, Slovakia; America: Canada, USA; Asia: China (Nelsen and Gargas 2006, 2008, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

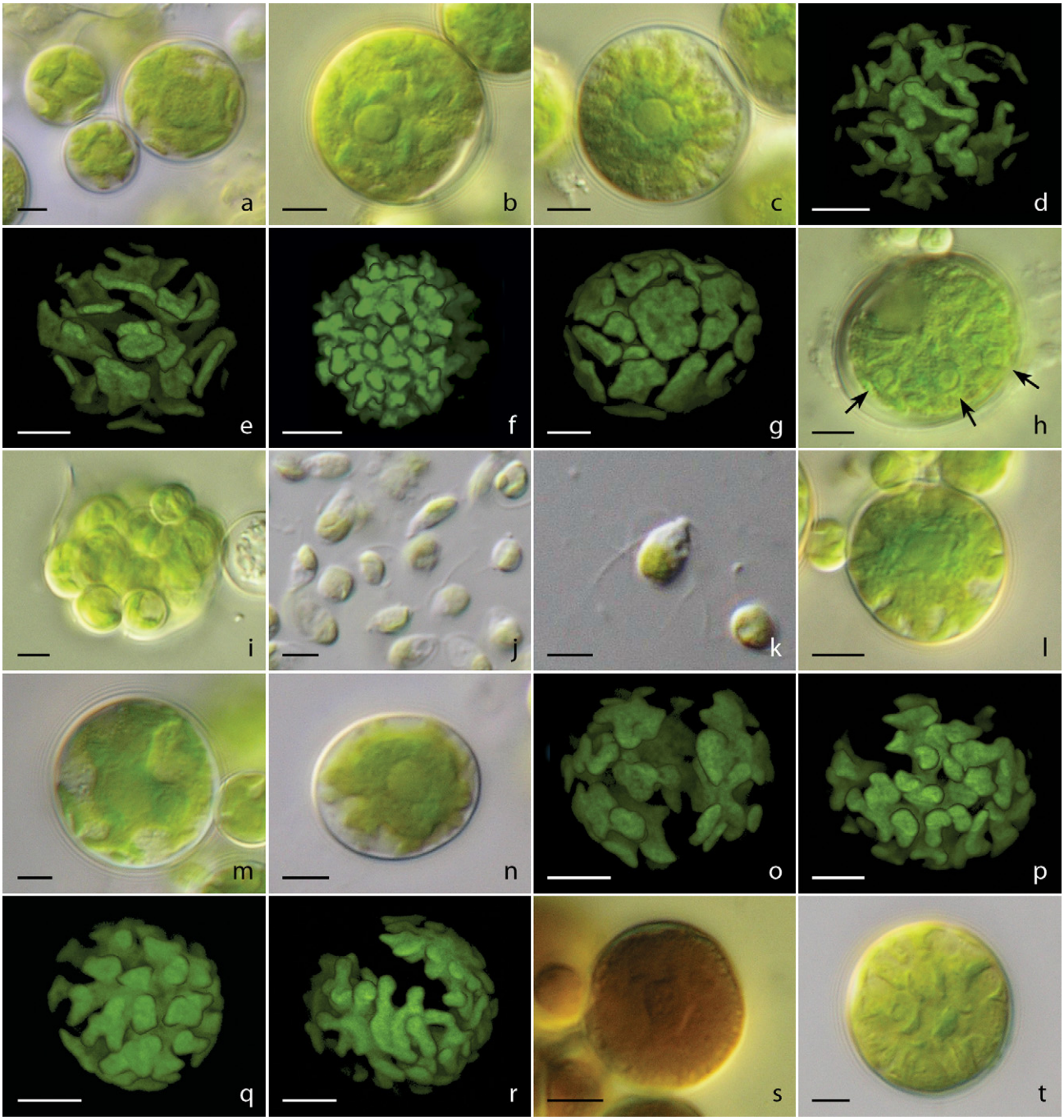


FIG. 7. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris woessiae* and *A. friedlii*. (a–k) *A. woessiae*. Light micrographs of young (a) and mature vegetative cells (b, c). Confocal reconstructions of deeply lobed (d), flat lobed (e), and crenulate (f) chloroplasts. (g) Flat terminations of chloroplast lobes. (h) Several pyrenoids (arrows) are formed within the chloroplast. (i) Aplanosporangium. (j) Zoospores. (k) Planozygote with four flagella. (l–t) *A. woessiae*. Light microscopy of shallowly lobed (l) and deeply lobed (m) vegetative cells. (n) Young vegetative cell with simple asteroid chloroplast. Confocal reconstructions of deeply lobed (o), shallowly lobed (p), crenulate (q), and parietal (r) chloroplasts. (s) Single budding pyrenoid. (t) Young aplanosporangium. Cells in Figure (s) stained by chloriodine solution; scale bar—5 μm.

*Ecology*: Mainly in ombrophobic lichens growing on acidic as well as basic substrates (Peksa and Škaloud 2011).

*Specificity*: With a single exception (*Cladonia*) found in thalli of lichen genus *Lepraria*.

***Asterochloris echinata*** Škaloud et Peksa **sp. nov.** (Fig. 8, a–i)

Vegetative cells always spherical, (5-)7–18(-21) μm in diameter (Fig. 8a). Cell wall thin, without any local thickenings. Young and mature cells exhibit

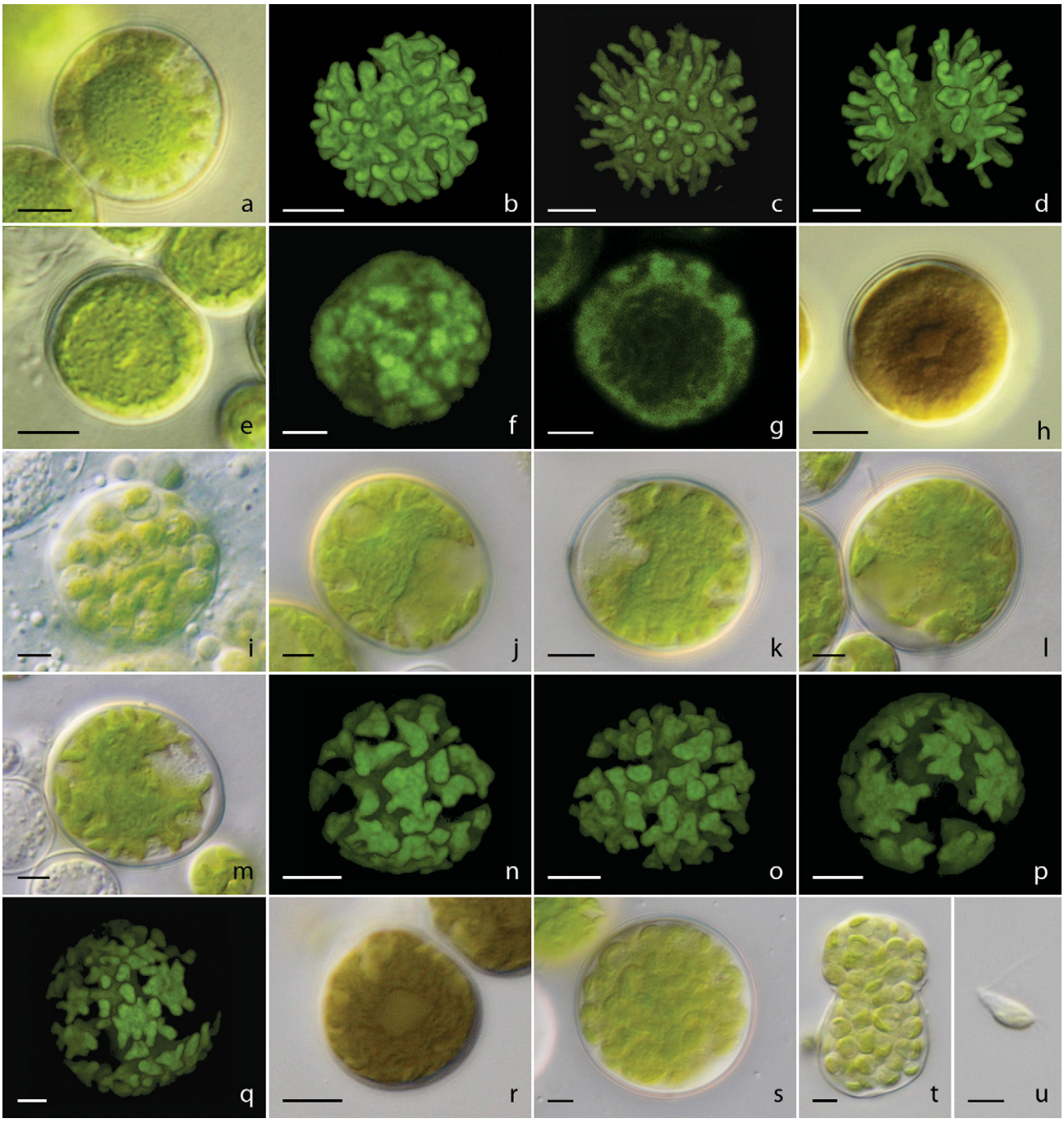


FIG. 8. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris echinata* and *A. lobophora*. (a–i) *A. echinata*. (a) Light micrograph of mature vegetative cell. Confocal reconstructions of crenulate (b) and echinate (c, d) chloroplast. Light micrograph (e) and confocal reconstruction (f) of globular chloroplast. (g) Confocal section through the globular chloroplast. (h) Several pyrenoids occur around the large central pyrenoid. (i) Aplanosporangium. (j–u) *A. lobophora*. Light micrographs of deeply lobed (j) and shallowly lobed (k) chloroplasts. (l) Vegetative cell with a flat local thickening of the cell wall. (m) Mature vegetative cell with the thickened cell wall. Confocal reconstructions of shallowly lobed (n), crenulate (o), and parietal (p, q) chloroplasts. A single pyrenoid is situated in the chloroplast lumen (r). (s) Mature aplanosporangium. (t) Liberated aplanospores. (u) Zoospore. Cells in Figure (h, r) stained by chloriodine solution; scale bar—5 μm.

central crenulate chloroplasts (Fig. 8b). In mature cells, the chloroplast often transform into the echinate form characterized by many thin radial lobes giving it a bristly appearance (Fig. 8, c and d). The crenulate chloroplast of old cells frequently transform into a highly specific, simple form without any lobes (Fig. 8, e and f). This form has a distinctive chloroplast ultrastructure in its central and marginal

regions. In the center, the starch accumulation causes the decrease in thylakoid numbers, and subsequent modification of the chloroplast's texture (Fig. 8g). The chloroplast contains from one to many distinct or indistinct pyrenoids. In the latter case, up to eight smaller pyrenoids are present in the vicinity of the central one (Fig. 8h). Sometimes, an indistinct granulation can be visible inside pyrenoids. Starch grains are embedded evenly throughout the chloroplast. Asexual reproduction by 64-128 aplanospores produced in spherical or ellipsoidal sporangia (Fig. 8i). Zoospores very rare, dorsiventrally flattened, 6 μm long and 4 μm wide.

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Peksa 186, deposited in the CAUP as the item TYPE-H 1012.

*Reference strains*: CAUP H 1012, SAG 2282.

*Type locality*: Phycobiont of *Lepraria rigidula*, collected on bryophytes on basalt rock, Klíč Mt., Lužické hory Mts, Czech Republic, September 18, 2004. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 186.

*Etymology*: The species epithet is named in reference to the echinate shape of chloroplast, which appeared in certain ontogenetic stages.

*Distribution*: So far known only from Europe: Bulgaria, Czech Republic, Portugal, Slovakia, Spain (Škaloud and Peksa 2010, Peksa and Škaloud 2011).

*Ecology*: Associates with ombrophilic lichens growing on acidic substrates (Peksa and Škaloud 2011).

*Specificity*: Found in the thalli of lichen genera *Lepraria* and *Cladonia*.

***Asterochloris lobophora*** Škaloud et Peksa **sp. nov.** (Fig. 8, j–u)

Vegetative cells usually spherical, occasionally oval and pyriform, 6–23(-25.5) μm in diameter (Fig. 8, j and k). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished (Fig. 8l). Very rarely, the cell wall is slightly thickened along its entire surface (Fig. 8m). Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells exhibit shallowly lobed chloroplast (Fig. 8n), sometimes transformed into the crenulate (Fig. 8o) or parietal form (Fig. 8, p and q). Even though the chloroplast can sometimes appear to be deeply lobed, the lobes never emerge directly from the pyrenoid surroundings. The chloroplast lobes can be terminated by all four known types, usually by finger-like or elongated extensions. The chloroplast contains single distinct granulated pyrenoid (Fig. 8r). Particularly prior to cell division, the pyrenoid sometimes divides into two parts. Starch grains are embedded either in a layer around the pyrenoid or evenly throughout the chloroplast. Asexual reproduction by 64–128 aplanospores or 128–256 zoospores produced in large spherical or slightly ellipsoidal sporangia (Fig. 8, s and t). Zoospores dorsiventrally flattened, 4–7 μm long and 2.5–3.5 μm wide, with posterior extensions (Fig. 8u).

*Holotype*: Cryopreserved photobiont cells isolated from the specimen Peksa 166, deposited in the CAUP as the item TYPE-H 1014.

*Reference strain*: CAUP H 1014.

*Type locality*: Phycobiont of *Lepraria caesioalba*, collected on siliceous rock, Kašperk Mt., Šumava Mts, Czech Republic, March 31, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 166.

*Etymology*: The species epithet is named in reference to the lobed chloroplast shape.

*Distribution*: Cosmopolitan, widely distributed. Europe: Czech Republic, Slovakia; America: Canada, USA; Asia: India (Piercey-Normore and DePriest 2001, Yahr et al. 2004, 2006, Cordeiro et al. 2005, Nelsen and Gargas 2006, Beiggi and Piercey-Normore 2007, Bačkor et al. 2010, Kotelko and Piercey-Normore 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011, Řídká et al. 2014).

*Ecology*: In temperate Europe, it prefers altitudes of about 500–1,000 m a.s.l; associates with ombrophilic lichens growing mainly on acidic substrates (Peksa and Škaloud 2011).

*Specificity*: Found in a number of lichen species belonging to genera *Cladonia*, *Lepraria*, *Diploschistes*, and *Stereocaulon*.

## DISCUSSION

*Species delineation in* Asterochloris. To date, a total of seven species are recognized within the genus *Asterochloris*. These species, established during the second half of the 20th century, were exclusively delimited based on morphological features such as cell size, cell shape, chloroplast morphology, cell wall thickness, and dissociation of aplanospores (Ahmadjian 1960, Archibald 1975, Tschermak-Woess 1980, Hildreth and Ahmadjian 1981). Each species formed a distinct, well-supported lineage in the concatenated ITS rDNA + actin phylogenetic tree (Fig. 2); however, our phylogenetic reconstruction points out the existence of several additional, well-supported lineages within the genus. Six of these new lineages were investigated in detail in this study. In general, we can apply two alternative taxonomic approaches to assess the observed genetic diversity. First, we can consider each genetic lineage to be a separate, distinct species and based on this approach, the genus *Asterochloris* would encompass tens, if not hundreds, of undescribed species. Second, genetic diversity can be considered to be a manifestation of substantial infraspecific variability. Applying this "large species" concept would involve merging all described species into a single species, namely *A. phycobiontica*.

The existence of a single, genetically divergent species is supported by the presence of identical SSU rDNA sequences in all of the investigated

strains and by the observation that the relatively low genetic variation in the ITS rDNA region is correlated with the absence of compensatory base changes (CBCs) among a number of *Asterochloris* lineages (Škaloud and Peksa 2010). In prokaryotes, it was proposed that species' boundaries should be defined by a fixed threshold of genetic divergence in SSU rDNA (Rosselló-Mora and Amann 2001). This concept considers that all strains showing a similarity higher than 97% belong to the same species. Applying this concept to *Asterochloris* would result in recognizing only a single species; however, this species concept was never applied to eukaryotic organisms, and its applicability to prokaryotes has been seriously criticized (Pedrós-Alió 2006, Stackebrandt and Ebers 2006). Similarly, the presence of CBCs in the conserved regions of the ITS2 molecule has been proposed to represent a threshold for defining species boundaries in eukaryotes (Coleman 2000); however, this concept has been subjected to mounting criticism (Caisová et al. 2011, 2013, Assunção et al. 2012). In particular, it was demonstrated that the CBCs are not diagnostic at the species level and that even genera, families, and orders of green algae can lack CBCs in such regions (Caisová et al. 2011, Škaloud and Rindi 2013). Indeed, a causal link between ITS2 secondary structure and speciation mechanisms in eukaryotes simply does not exist. Therefore, the presence and number of CBCs are most probably direct consequences of the accumulation of mutations during the evolutionary process, simply reflecting the genetic distance among organisms.

In this study and during our previous investigation of *Asterochloris* algae, we detected substantial genetic, morphological, and ecological differences among particular lineages. First, although the overall mean distance among the lineages was rather low in ITS rDNA (*P*-distance: 0.022), a considerable genetic differentiation was observed in the actin locus (*P*-distance: 0.168). Contrary to their broad utilization in fungal research (e.g., Grube and Kroken 2000, Daniel and Meyer 2003), actin intron sequences are still rarely used for species identification in protists. The potential of this marker for identifying and delimiting protist species has been demonstrated in photobiont genera *Asterochloris* (Nelsen and Gargas 2006, Škaloud and Peksa 2010) and *Trebouxia* (Kroken and Taylor 2000, Muggia et al. 2010) and in the heterotrophic chrysophycean genus *Spumella* Cienkowsky (Stoeck et al. 2008).

Second, our statistical analyses revealed a significant morphological differentiation of all investigated *Asterochloris* lineages. Although cell size was determined to be highly plastic under culture conditions, all other morphological features appear to well discriminate among species. Chloroplast morphology, in particular, could be considered to be the best morphological marker for species delineation. Škaloud and Peksa (2008) pointed to the existence of several specific chloroplast types occurring during species ontogeny. In the current study, we demonstrated that although these types are frequently shared by more than one species, particular species could be well recognized by the dominance and unique assemblage of particular types of chloroplasts (Fig. 4, b and c). For example, the parietal lobed chloroplast type prevails in *A. phycobiontica*, frequently occurs in *A. magna*, only occasionally appears in *A. lobophora*, and very rarely develops in *A. leprarii*, *A. woessiae*, *A. friedlii*, and *A. irregularis*. Moreover, some chloroplast types occur in a small minority of species and could be used to easily define these species. For example, the combination of crenulate and echinate chloroplast types occurs only in *A. echinata*, while the presence of a globular type defines *A. magna*. Interestingly, closely related lineages were generally similar in terms of morphology (i.e., *A. gaertneri–A. irregularis* and *A. phycobiontica–A. lobophora*).

Finally, previous molecular investigations detected clear ecological differentiation of *Asterochloris* lineages. Initially, Piercey-Normore and DePriest (2001) explained the photobiont switching among the symbiotic lichen associations as an analogy to human agriculture, where locally best adapted crops are selected and subsequently distributed. Later on, Piercey-Normore (2006) hypothesized that algal genotypes coexisting in the same lichen thalli may be adapted to different forest light levels. Yahr et al. (2006) then suggested the ecological specialization of photobiont lineages, depending on the local environment. Quite recently, specific ecological factors that drive the specialization of *Asterochloris* lineages were detected by Peksa and Škaloud (2011). Besides substrate and climatic preferences, exposure to rain and sun was the most significant environmental factor, clearly distinguishing particular lineages. The photobionts from ombrophobic and ombrophilic lichens were clustered into completely distinct clades.

In light of the above-mentioned genetic, morphological, and ecological inferences, we believe that each well-resolved *Asterochloris* lineage should be considered to be a distinct species. In fact, by applying an alternative, broader species concept, obvious differences among the lineages would be ignored. This would ultimately prevent us from understanding the true diversity, distribution, and specificity of *Asterochloris* species, as well investigating evolutionary processes that occur at the species level. Therefore, we described all lineages investigated in this study as new species.

*Biogeography and specificity of new* Asterochloris *species.* A comparison with previously published diversity surveys allows us to trace the biogeography and specificity of newly proposed *Asterochloris* species. Although the biogeography of microorganisms has become a highly discussed topic (Caron 2009), investigations dealing with the biogeography of

symbiotic protists are very scarce. Geographic separation of particular lineages has been reported for reef-coral dinoflagellate endosymbionts (e.g., Finney et al. 2010, LaJeunesse and Thornhill 2011), as well as for endosymbiotic green algae of the ciliate *Paramecium bursaria* (Hoshina et al. 2005). By contrast, population studies on lichenized *Trebouxia* species indicated that the distribution of particular genotypes is particularly shaped by either climatic factors (Fernández-Mendoza et al. 2011) or distribution patterns of mycobiont partners (Buckley et al. 2014).

The single study dealing with the biogeography of *Asterochloris* photobionts indicated generally wide (eurychoric) distribution of species (Řídká et al. 2014). Nevertheless, the habitat area of common lineages seems to be more or less restricted based on climatic preferences (e.g., warm-temperate to (sub) arctic distribution of *A. glomerata*). Though the real diversity of *Asterochloris* algae is still greatly undersampled, it seems that at least some lineages exhibit restricted geographic distribution independent of climatic factors. According to the actually available genetic data, three of the six newly proposed species (*A. echinata*, *A. gaertneri*, and *A. leprarii*) occur only in Europe (Fig. 2b). These species have never been reported from climatically analogous regions in eastern USA, though the investigations were performed on identical or closely related lichen species (Nelsen and Gargas 2008).

In lichen associations, the term "specificity" is used to refer the range of compatible partners for a given symbiont (Yahr et al. 2006). Lichen specificity is usually perceived from the perspective of a fungal partner, that is, as the range of possible photobionts for a given fungal species. In general, both fungal specialists (having a high specificity) and generalists (having a low specificity) have been distinguished in many lichen genera (see Muggia et al. 2014). However, the specificity could be conceived from the algal perspective, as well. Using our present data, we can compare the algal specificity toward the fungal genera of the newly described *Asterochloris* species. All species seem to be highly specific toward the genera *Cladonia* and *Lepraria* (Fig. 2b). Two species (*A. gaertneri* and *A. leprarii*) even form the symbiotic associations exclusively with the fungal genus *Lepraria*. Interestingly, though a number of *Stereocaulon* thalli have been investigated for the diversity of their algal partners (Piercey-Normore and DePriest 2001, Nelsen and Gargas 2006, Bačkor et al. 2010, Škaloud and Peksa 2010, own unpublished data), the species herein described as new are obviously not preferred by this fungal genus. In fact, *Stereocaulon* is much widely preferred by two closely related species *A. glomerata* and *A. irregularis* (Fig. 2a).

*Assessing species diversity in protists.* Estimation of the total species diversity in protists remains a highly controversial topic (Caron 2009). Global protist diversity has been proposed to be extraordinarily high by some (Foissner 1999) and generally much lower and fundamentally different from the biodiversity of macroorganisms by others (Fenchel and Finlay 2003). Accordingly, while Finlay and Fenchel (1999) estimated that there are approximately 20,000 protist species, others estimate that there are several million undescribed protist species (Pawlowski et al. 2012). Such substantial differences are primarily caused by differences in methodology (e.g., morphological vs. molecular approaches), species concept (see Boenigk et al. 2012), and theoretical framework (e.g., dispersal-gene flow paradox; De Meester et al. 2002); however, the vast majority of recent investigations have provided undeniable evidence that the overall species diversity of protists is greatly underestimated (e.g., Caron et al. 2012, Pawlowski et al. 2012).

During the past decade, analysis of SSU ribosomal RNA genes has become the most commonly used approach to investigate the diversity of protists. A number of studies have revealed an extremely high proportion of SSU rDNA sequences that could not be assigned to any described species (e.g., López-García et al. 2001, Behnke et al. 2011). Exploration of SSU rDNA sequences has often revealed the existence of several novel, highly diverse lineages (Dolven et al. 2007, Howe et al. 2009). More recently, technological progress in sequencing has enabled researchers to investigate protist diversity at previously unattainable scales. Using next-generation sequencing (NGS) technologies, thousands of SSU rDNA amplicon sequences can be produced from a single sample (Edgcomb et al. 2011); however, the sequence length obtained by NGS sequencing is (at the time of writing) insufficient to characterize complete SSU rDNA genes. Therefore, only short, hypervariable SSU rDNA regions are usually targeted to assess protist diversity (Dunthorn et al. 2012).

In NGS studies, sequence data are typically converted into operational taxonomic units (OTUs) based on sequence similarity; these units are often treated as being synonymous to species (Schmidt et al. 2014). Several methods have been developed to cluster SSU rDNA sequences into OTUs; however, they often partition sequence data differently (Sun et al. 2011). In addition, the taxonomic power of OTUs generated based on sequence similarities has been questioned (Boenigk et al. 2012). In the current study, we demonstrated that morphologically and ecologically diverse species can share identical SSU rDNA sequences. As a consequence, such species would be grouped into a single OTU by NGS data processing. Considering that closely related, mostly cryptic species of protists are commonly identical in their SSU rDNA sequences, the true diversity of eukaryotes can be much greater than that estimated by NGS data. In addition, organisms sharing high SSU rDNA sequence similarity can significantly differ in their ecology and distribution. Therefore, we suggest that the rapidly

evolving ITS region should be sequenced, in addition to the broadly used SSU rDNA gene, in NGS-based protist diversity investigations (e.g., Bachy et al. 2013).

*Sexuality of lichen photobionts.* Focusing on the main objective of this study, we performed a detailed investigation of the morphology and life cycle of many isolated photobionts, which involved hundreds of hours of microscopic observations. On two occasions, we had a brief opportunity to observe sexual reproduction in *Asterochloris woessiae* cultures, as evidenced by the fusing of biflagellate gametes. According to contemporary symbiotic dogma, lichen symbiosis should lead to the loss of sexual reproduction in the algal symbiont as a result of highly evolved and integrated symbiotic association (Law and Lewis 1983). The absence of sexual reproduction in lichen photobionts (except for the genus *Trentepohlia*) is, in fact, frequently mentioned in the literature (e.g., Ahmadjian 1987, Gärtner 1992, Friedl and Büdel 1996) and is interpreted as preventing the production of novel genotypes that would be less suited to the mycobiont (Ahmadjian 1993). However, records confirming sexual reproduction in photobionts do exist. The first exhaustive description of sexual reproduction in *Trebouxia* was published by Warén (1920), who observed frequent production of gametes and their subsequent fusion in photobionts of *Anaptychia ciliaris*, *Physconia distorta*, and *Xanthoria parietina*. Nine years later, Jaag (1929) reported sexual reproduction by iso- and anisogamy in a "*Cystococcus parmeliae*" photobiont isolated from *Flavoparmelia caperata*. Another observation was made by Ahmadjian (1959, 1960), who described frequent sexual reproduction in *Trebouxia impressa* isolated from *Physcia stellaris*. In both cases, isogamous sexual reproduction resulted in the formation of spherical, smooth-walled zygotes. Finally, indirect evidence of sexual reproduction in *Trebouxia* was presented by Kroken and Taylor (2000), who found a recombining population structure in photobionts of *Letharia* spp. by comparing ITS and actin sequences.

Although the existence of sexual reproduction has been disregarded, for example, by Gärtner (1985b), we consider that all of the abovementioned data, as well as our direct observation, clearly establish the presence of sexual reproduction in photobiont genera *Trebouxia* and *Asterochloris*. As sexual reproduction was previously reported only for *Trebouxia* s. str., gamete fusion in *A. woessiae* represents the first record of sexual reproduction in *Asterochloris*.

In conclusion, we demonstrated the obvious existence of a great number of distinct species within the genus *Asterochloris*, which differ genetically, morphologically, and ecologically. In general, the existence of an extraordinarily high number of cryptic, functionally differentiated species is probably the rule rather than the exception in protists. As a consequence, real species entities are poorly defined, providing little use in evaluating their distribution and diversity patterns or clarifying their ecological roles within ecosystems (e.g., Vyverman et al. 1996, Lilly et al. 2007). In addition, the existence of cryptic species with narrow ecological optima would significantly affect our strategies for conservation management (van Oppen and Gates 2006, Cotterill et al. 2008).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Table S1.** *Asterochloris* sequences used in phylogenetic reconstruction an haplotype networks, including strain and sample numbers, mycobiont species, geographic origin, and GenBank accession numbers for the ITS rDNA, actin, SSU rDNA, and rbcL loci.

**Table S2.** Morphological characteristics of investigated *Asterochloris* strains.