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TAXONOMIC REVISION AND SPECIES DELIMITATION OF COCCOID GREEN ALGAE CURRENTLY ASSIGNED TO THE GENUS *DICTYOCHLOROPSIS* (TREBOUXIOPHYCEAE, CHLOROPHYTA)[1]

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Coccoid green algae traditionally classified in *Dictyochloropsis* have a complex, reticulate chloroplast, when mature, without a pyrenoid. They occupy remarkably diverse ecological niches as free-living organisms or in association with lichen-forming fungi and were recently shown to form two distinct lineages within Trebouxiophyceae. We used a polyphasic approach to revise the taxonomy of the genus. Based on phylogenetic analysis of the 18S rRNA gene, and detailed morphological investigation using comparative conventional light and confocal microscopy, we have assigned these lineages to two genera, *Dictyochloropsis* and *Symbiochloris* gen. nov. We have reconsidered the diagnostic generic features as follows: *Dictyochloropsis* comprises only free-living algae with a reticulate chloroplast, forming lobes in a parallel arrangement at some ontogenetic stages, and which reproduce only by means of autospores. This agrees with Geitler's original diagnosis of *Dictyochloropsis*, but not with the later emendation by Tschermak-Woess. Consequently, the species of *Dictyochloropsis* sensu Tschermak-Woess are assigned to *Symbiochloris*, with new combinations proposed. *Symbiochloris* encompasses free-living and/or lichenized algae with lobed chloroplasts and that reproduce by forming zoospores characterized by two subapical isokont flagella that emerge symmetrically near the flattened apex. In addition, using coalescent-based approaches, morphological characters and secondary structure of ITS transcripts, we inferred species boundaries and taxonomic relationships within the newly proposed genera. Two species of *Dictyochloropsis* and nine species of *Symbiochloris* are delimited, including the newly described species *D. asterochloroides*, *S. handae*, *S. tropica*, and *S. tschermakiae*. Our results further support the non-monophyly of autosporine taxa within Trebouxiophyceae.

Key index words: **chloroplast; cryptic species; morphology; phylogeny; species boundaries**

***Abbreviations*: 3NBBM, Bold's Basal Medium with three times the nitrate; AOBS, Acousto Optical Beam Splitter; CAUP, Culture Collection of Algae of the Charles University in Prague, Czech Republic; CIPRES, Cyberinfrastrucure for Phylogenetic Research; ITS, internal transcribed spacer; NIES, Microbial Culture Collection at the National Institute for Environmental Studies; *rbc*L, ribulose-bisphosphate carboxylase; SAG, Culture Collection of Algae at the University of Gottingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, USA**

The identification of coccoid green algae often presents a challenge for both ecologists and algal taxonomists due to the scarcity of visual characters suitable for diagnostic purposes and the lack of DNA sequence information. Among terrestrial green algae the genus *Dictyochloropsis* Geitler emend. Tschermak-Woess is relatively easy to identify by its distinctive, reticulate, parietal, chloroplast in young cells that, as the cells age, may become multilayered with lobes extending inwards toward the centrally located nucleus (Geitler 1966, Tschermak-Woess 1980, 1984, Ettl and Gärtner 1995, Škaloud et al. 2005). Species

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of *Dictyochloropsis* are reported from aerophytic substrata or soil (e.g., Mikhailyuk 2008, Neustupa and Škaloud 2008, Škaloud 2009) and also occur frequently as lichen symbionts (e.g., Tschermak-Woess 1984, Peršoh et al. 2004, Friedl and Büdel 2008, Werth and Scheidegger 2012, Dal Grande et al. 2014). To distinguish species, differences in chloroplast structures mainly are used (Tschermak-Woess 1984, Škaloud et al. 2005). Two major chloroplast types were recognized by Tschermak-Woess (1984): (i) the parietal chloroplast consisting of a peripheral network of interconnected lobes, and (ii) the multilayered chloroplast formed by several interconnected layers of lobes penetrating into the cell interior, sometimes approaching the central nucleus. Škaloud et al. (2005) recommended the use of confocal microscopy to better reveal the three-dimensional structure of the chloroplast.

Additional diagnostic features are differences in the size and shape of vegetative cells (Nakano and Isagi 1987) as well as asexual reproduction (Tschermak-Woess 1984). In addition to motile zoospores, two types of nonflagellated spores are distinguished in *Dictyochloropsis*: (i) autospores, i.e., larger spores (4–5 μm in diameter), 4–32 per sporangium, similar in appearance to young vegetative cells (Geitler 1966), and (ii) aplanospores, i.e., small spores (2.5–4 μm in diameter) produced in high numbers per sporangium, often considered as arrested zoospores (Tschermak-Woess 1980, 1995).

A faster and more accurate alternative for species identification is provided by DNA sequence comparisons. In contrast to morphology, molecular phylogenies also allow researchers to infer the phylogenetic relationships among algal species. This is particularly important in coccoid green algae because their simple morphology often hides an unexpected larger genetic diversity and may render a morphologically defined genus a polyphyletic assemblage (e.g., *Chlorella* Beijerinck; Huss et al. 1999, Krienitz et al. 2004, Leliaert et al. 2014). Also, the large number of 18S ribosomal RNA (SSU) gene sequences now available for green algae in public databases enables us to study green algal diversity without time-consuming culturing by establishing and separating SSU sequences directly from environmental samples. Such a culture-independent approach has frequently revealed new lineages of still unidentified organisms for which no cultures are available and thus also no morphological features known (e.g., Pace 1997, Diez et al. 2001, Lopez-Garcia et al. 2001, Moon-van der Staay et al. 2001). While this has been an important advance in understanding diversity at the level of major protistan clades, sequence information is even more useful for understanding species-rank diversity, where a larger number of reference sequences is essential, but, at present, may still be insufficient.

Recently, molecular investigations based on 18S rDNA and *rbc*L cpDNA sequences, as well as ITS secondary structures, showed that algal strains and lichen symbionts assigned to *Dictyochloropsis* rendered the genus polyphyletic (Škaloud et al. 2007, Dal Grande et al. 2014) and that the two clades might well represent distinct genera.

In this study, we present a taxonomic revision of strains assigned to *Dictyochloropsis*. We used coalescent-based methods of species delimitation to investigate species diversity in 21 strains representing all currently recognized species and several unidentified strains of aerial and symbiotic algae of *Dictyochloropsis* clade I and II sensu Dal Grande et al. (2014). In addition, we undertook a polyphasic approach, including a re-investigation of chloroplast features using confocal microscopy, to establish phylogenetic species.

MATERIALS AND METHODS

Sampling, isolation and culturing. The 21 cultivated strains used in this study were obtained from public culture collections: the Culture Collection of Algae at the University of Göttingen, Germany (SAG), the Culture Collection of Algae of Charles University in Prague (CAUP), and the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX). A set of nine strains of coccoid green algae with *Dictyochloropsis* morphology isolated from various aeroterrestrial habitats and lichens were provided by Drs Nobutaka Hanagata (University of Tokyo, Tokyo, Japan) and Shunji Takeshita (Hiroshima University, Hiroshima, Japan) and accessioned by the SAG culture collection under the strain numbers as listed in Table 1. In addition, we investigated tree bark material from different experimental forest plots as a part of an initiative to advance biodiversity research in Germany (www.biodiversity-exploratories.de). Tree bark was collected in the two biodiversity exploratories, Hainich National Park (south of Mühlhausen in Thuringia) and Swabian Alb ("Schwäbische Alb," surrounding the town of Münsingen in Baden-Württemberg). The bark samples were from beech trees (*Fagus sylvatica*) in the Hainich plots HEW06 (51° 26′ N, 10° 24′ E; age class forest, old timber, at 18 m height), HEW08 (51° 35′ N, 10° 51′ E; selection cutting forest, 0–2 m height) and HEW11 (51° 10′ N, 10° 40′ E; beech natural forest, old timber, at 0–2 m [R1] and 19 m [R2] height). In the Swabian Alb Biosphere Area, they were from spruce (*Picea abies*) in plot AEW03 (48° 41′ N, 9° 35′ E; spruce age class forest, young timber, at 0–2 m height,) and from beech in plot AEW08 (48° 38′ N, 9° 38′ E; extensively managed beech forest, at 0–2 m height). The samples were used for DNA extraction, rDNA cloning, and sequencing (see below) except for the sample from the plot AEW03 that was used for culturing. Here, biofilm material covering the tree bark was scraped off with a sterile scalpel, placed into a 1.5 mL reaction tube to which 500 μL sterile water was added and briefly vortexed. 100 μL of the suspension were then plated on agarized 3NBBM+V culture medium and kept at 20°C under culture conditions as described below. As soon as the first colonies appeared on the agar plate, they were removed with a sterile wooden stick and plated onto fresh agar plates. The culture was further purified by repeated transfers and plating on agarized culture medium. It has been accessioned by the SAG culture collection as strain SAG 2305. All cultures were maintained on agar slants (1.5%) at 18°C under a 14:10 light:dark regimeat a photon flux rate of about 25 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} from white fluorescent bulbs.

TABLE 1. List of studied strains, their new and previous species assignments, origins, references, and sequence accession numbers.

Actual species assignment	Original species assignment	Strain designation	Origin and reference	18S	*rbcL*	ITS
Dictyochloropsis asterochloroides	*D.* sp.	SAG 2073	Japan, rock; Handa (no. 538a)	GU017648	KC333605	–
	D. sp.	SAG 2098	Japan, concrete; Handa (no. 927i)	GU017664	KC333606	–
Dictyochloropsis splendida	*D. splendida*	CAUP H8601	Czech Republic, soil; Škaloud et al. (2005)	GU017662	KC333607	KC333515
	D. splendida	SAG 2071	Japan, rock; Handa (no. 892e)	GU017649	KC333609	KC333510
	D. splendida	SAG 2097	Japan, rock; Handa (no. 923e)	GU017661	KC333608	KC333511
	D. splendida	SAG 2305	Germany, Baden-Wuerttemberg, tree bark; Pahlmann	GU017658	–	KC333522
Symbiochloris irregularis	*D. irregularis*	SAG 2036	Austria, tree bark; Tschermak-Woess (2000)	GU017659	KC333600	–
	D. irregularis	SAG 2154	Japan, leaf surface; Handa (no. 906d)	GU017671	–	–
Symbiochloris pauciautosporica	*D. symbiontica* var. *pauciautosporica*	SAG 12.86	Spain, Teneriffe, photobiont of lichen *Crocodia aurata*; Tschermak-Woess (1984)	GU017651	–	–
	D. splendida	UTEX 2599	USA, photobiont of lichen *Phlyctis argena*; Tschermak-Woess (1995)	GU017665	EF113435	–
	D. splendida	UTEX 2612	USA, photobiont of lichen *Phlyctis argena*; Tschermak-Woess (1995)	GU017660	–	–
Symbiochloris reticulata	*D. reticulata*	SAG 53.87	Spain, Teneriffe, photobiont of lichen *Lobaria pulmonaria*; Tschermak-Woess	GU017650	KF960688	KC333513
Symbiochloris symbiontica	*D. symbiontica*	SAG 27.81	Austria, photobiont of lichen *Chaenothecopsis consociata*; Tschermak-Woess (1980)	GU017644	–	KC333512
	D. symbiontica	SAG 2070	Japan, lichen surface; Handa (no. 890p)	GU017645	–	–
	D. symbiontica	SAG 2099	Japan, lichen surface; Handa (no. 865d)	GU017646	–	–
Symbiochloris tropica	*D. reticulata*	CAUP H8602	Malaysia, tree bark; Škaloud et al. (2005)	GU017655	KC333603	KC333516
Symbiochloris handae	*D. reticulata*	SAG 2150/ CCHU5616	Japan, photobiont of lichen *Brigantiaea ferruginea*; Takeshita (1991)	Z47207	KC333590	KC333524
Symbiochloris tschermakiae	*D. symbiontica*	SAG 46.85	New Zealand, photobiont of *Crocodia aurata*; Tschermak-Woess (1984)	GU017654	KC333574	KC333521
Symbiochloris sp.	*D.* sp.	SAG 2069	Japan, rock; Handa (no. 706c)	GU017652	KC333601	KC333509
	D. splendida	SAG 244.80	Austria, photobiont of lichen *Chaenotheca brunneola*; Tschermak-Woess (1978a,b)	GU017653	KC333599	KC333514
	D. symbiontica	CAUP H8603	Malaysia, tree bark; Škaloud et al. (2005)	GU017663	KC333602	KC333517

Microscopy. To obtain a detailed morphological characterization of cultured *Dictyochloropsis* strains, we investigated them by both conventional light and confocal microscopy. Light microscopy observations were performed using Olympus BX51 and BX60 microscopes (Olympus Corp., Tokyo, Japan) equipped with differential interference contrast. Micrographs were taken with the Olympus BX60 microscope with an attached ColorView III camera (Soft Imaging System GmbH, Münster, Germany) and processed with the Cell^D image program (Soft Imaging System GmbH). For confocal microscopy, 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 5-month period of culturing, to well characterize the overall morphological variability. Zoospore formation was induced by transferring the cultures to a 1% glucose solution.

DNA extraction, PCR amplification, cloning, and sequencing. The strains for which 18S rDNA sequences were determined in this study are listed in Table 1. DNA was extracted from fresh cultures after breaking the cells with glass beads in a MinibeadbeaterTM cell homogenizer (Biospec, Bartlesvilles, OK, USA) and then using an Invisorb® Spin Plant Mini DNA extraction kit (Invitek, Berlin, Germany) as in Mikhailyuk et al. (2008). For extraction of DNA from environmental samples, the green biofilm covering tree bark was scraped off with scalpel and then stored in sealed bags previous to examination in the laboratory. Nuclear-encoded 18S rDNAs were amplified using primers NS1 and 18L (Hamby et al. 1988). PCR amplification was performed using reagents and a cycle as in Mikhailyuk et al. (2008). The positive PCR products were pooled, purified using the NucleoSpin-Extract-Kit (Macherey-Nagel, Düren, Germany) and eluted in 30 μL

elution buffer provided by the kit. For establishing clone libraries from environmental samples, the 18S rDNA was amplified from DNA extracts using primers NS1 and 18L, and the purified PCR products were ligated into the TOPO® vector (TOPO TA Cloning Kit; Invitrogen, Karlsruhe, Germany) and cloned into competent *E. coli* cells (Top10; Invitrogen). For insert check, positive clones were directly used as template for PCR using the same primers and amplification conditions as in the first PCR. Plasmid DNA was directly used for sequencing after extraction from positive clones (NucleoSpin-Extract-Kit; Macherey-Nagel), i.e., those with an insert of expected size which were cultured overnight in Lid-Bac reaction tubes (Qiagen, Hilden, Germany) containing Luria-Bertani medium and Ampicillin. Cycle sequencing was performed using the Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) as in Mikhailyuk et al. (2008) using a set of nested standard primers (Elwood et al. 1985). For checking clone libraries from environmental samples, a single sequencing reaction with the primer 895R (5′-AAATCCAAGAATTTCACCTC-3′) was used that covered a large portion including highly variable regions of the 5′-end of the 18S rRNA gene. Sequencing reactions were separated on an ABI Prism 3100 (Applied Biosystems) automated sequencer, and sequences were assembled using the program SeqAssem (Hepperle 2004) and are available in GenBank under the accession numbers GU017644-GU017674.

Sequences of the plastid-encoded large subunit of the ribulose-bisphosphate carboxylase-RuBisCO gene (*rbc*L) for 10 algal strains were obtained from GenBank (Table 1). In addition, for the species delimitation analysis we included 18S rDNAs and *rbc*L sequences from 18 lichen photobionts described in Dal Grande et al. (2014) if both loci were available for a given strain (Table 1).

Sequence alignment and phylogenetic analyses. The newly determined 18S rDNA sequences were compared with a broad selection of corresponding sequences from members of the Streptophyta and Chlorophyta. This also included the newly determined sequence for the lichen photobiont genus *Asterochloris* Tschermak-Woess, strain SAG 26.81 (authentic strain). The selection of sequences shown in Figure 1 was based on a phylogenetic tree comprising an expanded sample of more than 1500 rDNA sequences from green algae that is available in the 18S rDNA sequence database maintained in the ARB program (version 05.05.26, Ludwig et al. 2004, www.arb-home.de). This database was updated with all currently available 18S rDNA sequences from Chlorophyta and Streptophyta. The newly determined sequences (including the partial sequences from clone libraries) were added to the database using the parsimony interactive tool in ARB. The alignment was refined by comparing the sequences with their next relatives from the ARB database based on their pairing across a helix using secondary structure models as implemented in ARB. A subset of these sequences comprising a total of 70 representatives of the green algal class Trebouxiophyceae and two prasinophytes as outgroup was then downloaded from the ARB database for further analyses using the 50% base frequency filter. The 18S rDNA sequence data set (Appendix S1 in the Supporting Information) was subjected to Bayesian and maximum likelihood (ML) analyses with the softwares MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003), and RAxML (Stamatakis 2014). Model selection for the Bayesian approach, number of rate categories, proportion of invariable sites, and the gamma distribution parameter were determined with JMODELTEST v3.7 (Guindon and Gascuel 2003, Darriba et al. 2012). For Bayesian analysis, the 010230 + I+G+F model was selected by the corrected Akaike criterion (AIC) with estimations of nucleotide frequencies (A = 0.2358, C = 0.2433, G = 0.2791, T = 0.2419), a rate matrix with six different substitution types, assuming a heterogeneous rate of substitutions with a gamma distribution of variable sites (number of rate categories = 4, shape parameter $\alpha = 0.4890$) and a proportion of invariable sites (pinvar) of 0.4200. The Bayesian analysis was performed using four Markov chains and 5,000,000 generations sampling every 100 generations with the first 25% of the sampled trees discarded, leaving 12,500 trees. Posterior probabilities (PP) were then calculated from two independent runs using the 50% majority rule consensus of the kept trees. ML searches were performed using RAxML on the CIPRES server with gamma-distributed rate variation and a proportion of invariant sites, and 1,000 bootstrap (BS) replicates (Miller et al. 2010).

We tested whether observed branch length differences between *Dictyochloropsis* clade I and II were the result of a significant departure from rate constancy using Baseml (PAML 3.14 b package; Yang 2007). A molecular clock was used as null model, while in the alternative model each branch was allowed its own unique rate of evolution. These two models were contrasted for each gene partition (18S, *rbc*L; Appendix S2 in the Supporting Information) separately across the two-locus phylogeny, assuming a GTR+G model. We compared the resulting likelihoods using a likelihood ratio test.

Multi-locus coalescent species delimitation. For testing the species boundaries, *Dictyochloropsis* clade 1 and clade 2 two-locus data sets (18S and *rbc*L) were analyzed separately. *Dictyochloropsis* clade 1 data set included six 18S (Appendix S3 in the Supporting Information) and five *rbc*L (Appendix S4 in the Supporting Information) sequences for a total of six algal strains. *Dictyochloropsis* clade 2 included 32 18S (Appendix S5 in the Supporting Information) and 31 *rbc*L (Appendix S6 in the Supporting Information) sequences for a total of 32 algal strains.

For both data sets, well-supported (BS = 75%, PP = 0.95) monophyletic clades from ML and Bayesian phylogenies were taken as putative species, resulting in a 3-species scenario for *Dictyochloropsis* clade 1 (Fig. 2) and in a 12-species scenario for *Dictyochloropsis* clade 2 (Fig. 3). The marginal posterior probability of these scenarios was estimated using the program BP&P v3 that incorporates phylogenetic uncertainty in a Bayesian framework while eliminating the need for a fixed, user-specified guide tree (Yang and Rannala 2014). The program in fact takes into account uncertainty of gene tree topology and branch lengths and proposes changes in the guide species tree using the nearest-neighbor interchange algorithm. A gamma prior G (1, 10), with mean 1/10 = 0.1 (one difference per 10 bp) was used on the population size parameters (s). The age of the root in the species tree ($\tau 0$) was assigned the gamma prior G (2, 2000), while the other divergence time parameters were assigned the Dirichlet prior. Starting guide species-trees were generated using a coalescent-based hierarchical Bayesian model using *BEAST 2.1 (Drummond and Rambaut 2007, Jones 2014), with Birth–Death process and gamma-distributed population sizes for the species tree prior and a pair-wise linear population size model with a constant root.

Species boundaries in the group were also tested using STACEY as implemented in BEASTv2.2 (Jones 2014) by searching all combinations among individuals in the two data sets, using Birth–Death process and gamma-distributed population sizes for the species tree prior and a pair-wise linear population size model with a constant root, for 20 M generations and 20% burn-in. We selected the best locus-specific model of evolution for each gene using JMODELTEST v3.7. We performed cluster analyses using SpeciesDelimitationAnalyser (Jones et al. 2015).

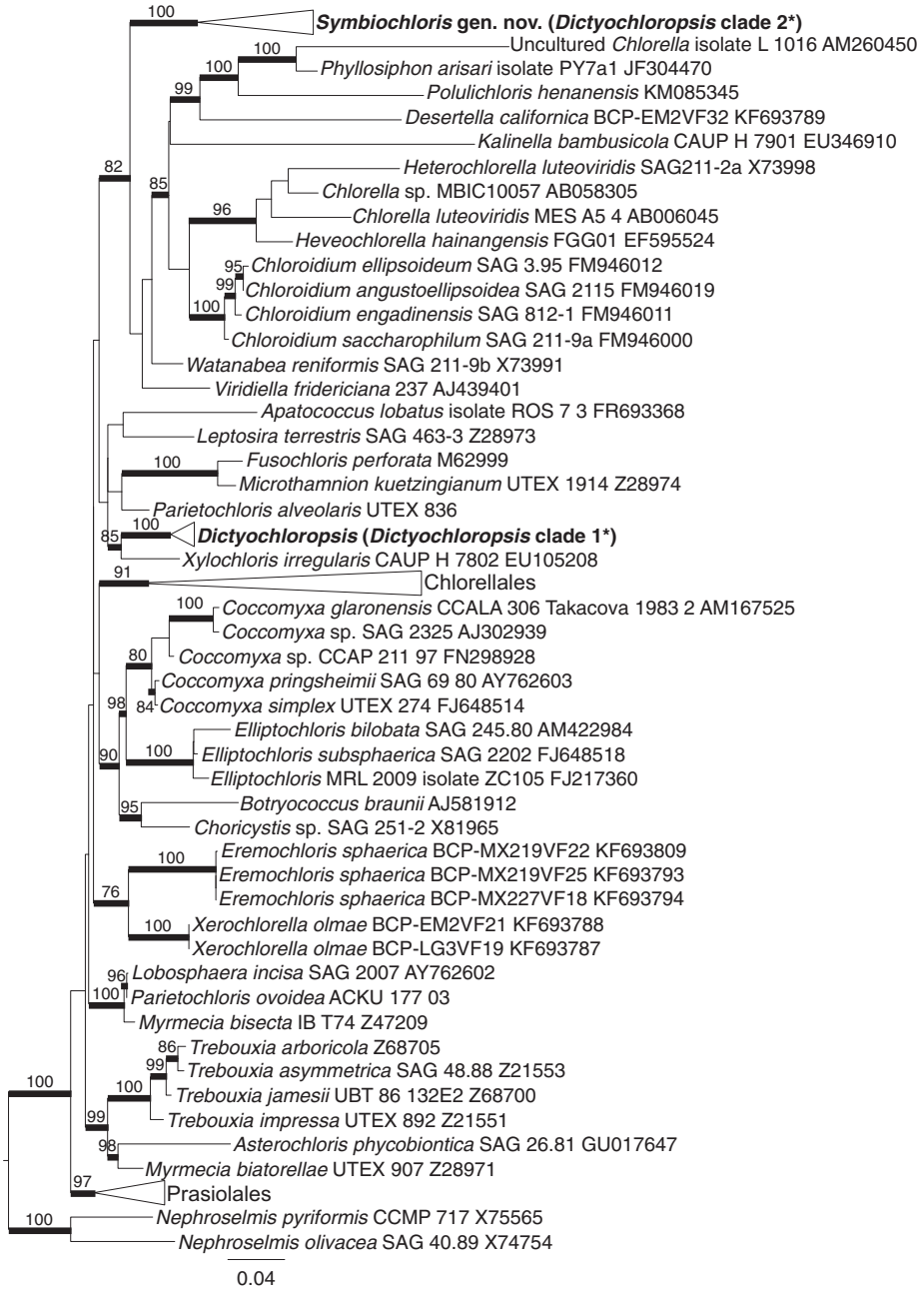

FIG. 1. Maximum likelihood (ML) tree of strains of aeroterrestrial green algae previously assigned to *Dictyochloropsis* and other members of the green algal class Trebouxiophyceae inferred from 18S rRNA gene sequences (1,879 bp long; 573/387 variable/parsimony informative sites). Some of the clades were collapsed for clarity of presentation. Sequences representing the *Chlorellales*- and *Prasiola*-Clades (Karsten et al. 2005) are listed in Table S1 in the Supporting Information. Numbers above branches indicate ML bootstrap proportions (>75%). Thickened branches indicate Bayesian Markov chain Monte Carlo posterior probabilities >0.95. The two prasinophytes *Nephroselmis olivacea* and *N. pyriformis* (Pseudoscourfieldiales) served as outgroup taxa. Asterisks refer to the names under which the genera were reported in Dal Grande et al. (2014).

ITS secondary structures. Most likely ITS2 secondary structures of the RNA transcript were determined by depicting the highly conserved start and end region of the four helices (Mai and Coleman 1997). The structure of these sequence sections has been calculated using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

RESULTS

Phylogenetic analyses. We obtained 21 new 18S rRNA gene sequences of green algae assigned to *Dictyochloropsis*. A total of 92 taxa were included in the 18S rRNA gene phylogeny, for a total alignment length of 1879 sites. *Dictyochloropsis* was clearly polyphyletic and occurred in two well-supported monophyletic groups as reported by Dal Grande et al. (2014), namely *Dictyochloropsis* clade 1 and *Dictyochloropsis* clade 2. The position of the *Dictyochloropsis* clade 1 lineage within the Trebouxiophyceae remained ambiguous, although we found moderate support for a sister relationship with the tropical strain *Xylochloris irregularis* Neustupa, M. Eliáš & Škaloud (Fig. 1). *Dictyochloropsis* clade 2 was inferred in the basal position of the *Watanabea*-clade sensu Karsten et al. (2005), comprising several lineages of autosporine coccoid green algae. *Viridiella fridericiana* Albertano, Pollio & Taddei and *Watanabea reniformis* Hanagata, Karube, Chihara & P.C. Silva were resolved as the next closest relatives.

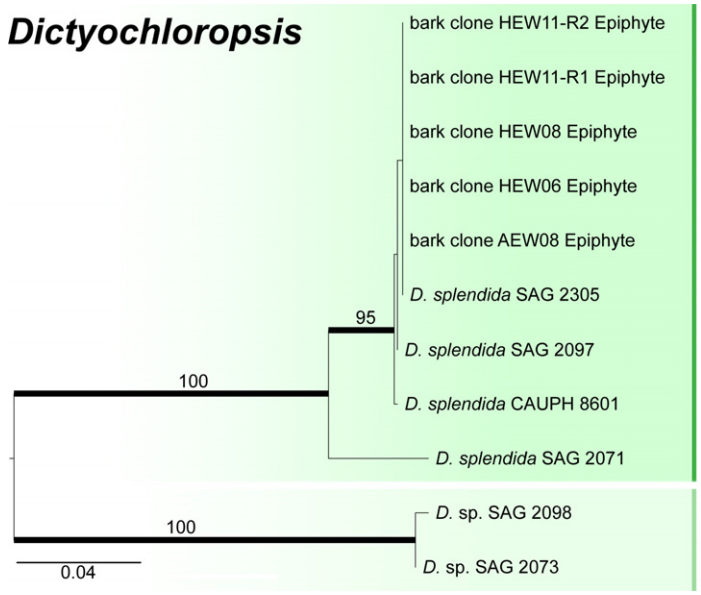

FIG. 2. Maximum likelihood (ML) tree of *Dictyochloropsis* based on a concatenated 2-locus data set including 18S and *rbcL* sequences. Numbers above branches indicate ML bootstrap proportions (>75%). Thickened branches indicate Bayesian Markov chain Monte Carlo posterior probabilities >0.95. Boxes and shading indicate putative species.

The individual gene tree phylogenies included 11 and 33 strains of *Dictyochloropsis* clade 1 and 2, respectively (Figs. 2 and 3). The two-locus data sets yielded well-supported topologies for both clades.

Dictyochloropsis clade 1 included only free-living algae. Strain CAUP H8601, previously identified as *D. splendida* Geitler (Škaloud et al. 2005), formed a well-supported lineage together with the strains isolated from a tree bark (SAG 2071, SAG 2097, SAG 2305), as well as all tree bark environmental clones (AEW08, HEW06, HEW08, HEW11-R1, HEW11-R2). The Japanese strains SAG 2073 and SAG 2098 formed a closely related sister lineage with *D. splendida* (Fig. 2).

Dictyochloropsis clade 2 included both symbiotic and free-living algae (Fig. 3). *D. splendida* as well as *D. symbiontica* Tschermak-Woess and *D. reticulata* (Tschermak-Woess) Tschermak-Woess were paraphyletic within this clade; *D. irregularis* T.Nakano & Isagi was the only species based on previous identifications by morphology that also was found monophyletic in the 18S-*rbc*L phylogeny. Strains previously assigned to *D. splendida*, (i.e., UTEX 2599, UTEX 2612, SAG 2069 and SAG 244.80) were distributed in three different lineages. Strains UTEX 2599 and UTEX 2612 formed a well-supported lineage with authentic strain *D. symbiontica* var. *pauciautosporica* Tschermak-Woess SAG 12.86 and two lichen photobionts from Brazil (MPN124-167). Strain SAG 2069 was a sister lineage to *D. irregularis*. Strain SAG 244.80 formed an independent lineage within the clade. Two Japanese strains SAG 2070 and SAG 2099 formed a well-supported clade with the authentic strain *D. symbiontica* SAG 27.81. Interestingly, *D. symbiontica* was most basal, i.e., it formed a sister-group with all other strains of *Dictyochloropsis* clade 2. The species was separated by a long branch corresponding to a rather high number of synapomorphic sequence differences from other strains of the clade. In contrast to its previous assignment, strain SAG 46.85 was not closely related with *D. symbiontica*, but formed a well-supported clade together with three photobionts of *Crocodia aurata*, one photobiont of *Lobaria patinifera* and one photobiont of an unidentified *Pseudocyphellaria* from Brazil (MPN169). This clade was sister to the one including SAG 53.87 and other closely related symbiotic algae found in the lichen genera *Dendriscosticta*, *Lobaria*, and *Ricasolia*. Strain SAG 2150, also isolated from a lichen, was neither closely related to the former strains nor to any other member of *Dictyochloropsis* clade 2. Strains CAUP H8602 and CAUP H8603 formed another well-supported lineage that occupied a rather basal position within the clade. In contrast to their previous identifications (Škaloud et al. 2005) both strains were not closely related with other *D. reticulata* strains and with the authentic strain *D. symbiontica* SAG 27.81, respectively.

We found significant differences in branch lengths between the two *Dictyochloropsis* clades ($\chi^2(1) = 84.47$, df = 36, $P < 0.001$). *Dictyochloropsis* clade 2 taxa had consistently longer branches than clade 1 representatives, suggesting an accelerated evolutionary rate in *Dictyochloropsis* clade 2.

Microscopy. Molecular differentiation of *Dictyochloropsis* strains was also clearly reflected in morphological characters. All strains belonging to *Dictyochloropsis* clade 1 shared the lamellate structure of the chloroplast. Especially in mature cells, the chloroplast consisted of many interconnected lamellae that often occur in parallel and form a highly arranged structure at the chloroplast periphery (Fig. 4), as previously described in detail for strain *D. splendida* CAUP H8602 (Škaloud et al. 2005). In young cells, the network of chloroplast lobes was one-layered and parietal, i.e., it was closely

FIG. 3. Maximum likelihood (ML) tree of *Symbiochloris* based on a concatenated two-locus data set including 18S and *rbcL* sequences. Numbers above branches indicate ML bootstrap proportions (>75%). Thickened branches indicate Bayesian Markov chain Monte Carlo posterior probabilities >0.95. Boxes and shading indicate putative species.

appressed to the cell wall (Fig. 4a). The four closely related strains CAUP H8601, SAG 2071, SAG 2097, and SAG 2305 were characterized by the formation of large spherical cells reaching 20(-30) µm in diameter (Fig. 4b). In these cells, chloroplast lobes were usually arranged in parallel (Fig. 4, c and d). The strains reproduced exclusively by (4-)8–16 autospores each ~8–15 µm in diameter (Fig. 4e) that were liberated by rupturing of the mother cell wall (Fig. 4f). The Japanese strains SAG 2073 and SAG 2098 had specific chloroplast features in common that made them clearly distinct from the four previously described strains. In young cells the chloroplast was central and stellate (Fig. 4g), in contrast to the flattened parietal chloroplast described above (Fig. 4a). Sometimes also in mature cells the arrangement of chloroplast lobes had a stellate appearance (Fig. 4h) and tubular apertures occurred inside of the chloroplast as seen by confocal microscopy (Fig. 4i). However, both strains shared the formation of chloroplast lobes oriented in parallel with other strains of *Dictyochloropsis* clade 1 (Fig. 4, j and k). Reproduction of both strains was exclusively by 4–16 autospores (Fig. 4l).

Other strains previously assigned to *D. splendida*, i.e., those strains belonging to *Dictyochloropsis* clade 2, clearly differed from the non-lichenized *D. splendida* strains in their chloroplast features and asexual reproduction (Figs. 5–7); they had a chloroplast exhibiting a regular, net-like, lobate appearance in surface view (Fig. 5c). The structure of interconnected lamellae arranged in parallel at the chloroplast periphery, characteristic for members of *Dictyochloropsis* clade 1, was absent. The frequent formation of "aplanospores" sensu Tschermak-Woess (1980), i.e., a large number (32–128) of immotile small daughter cells with cell walls (Figs. 5k and 7n) and/or zoospores with subapical, distantly inserted isokont flagella (Fig. 7f) defined members of the *Dictyochloropsis* clade 2, while the production of

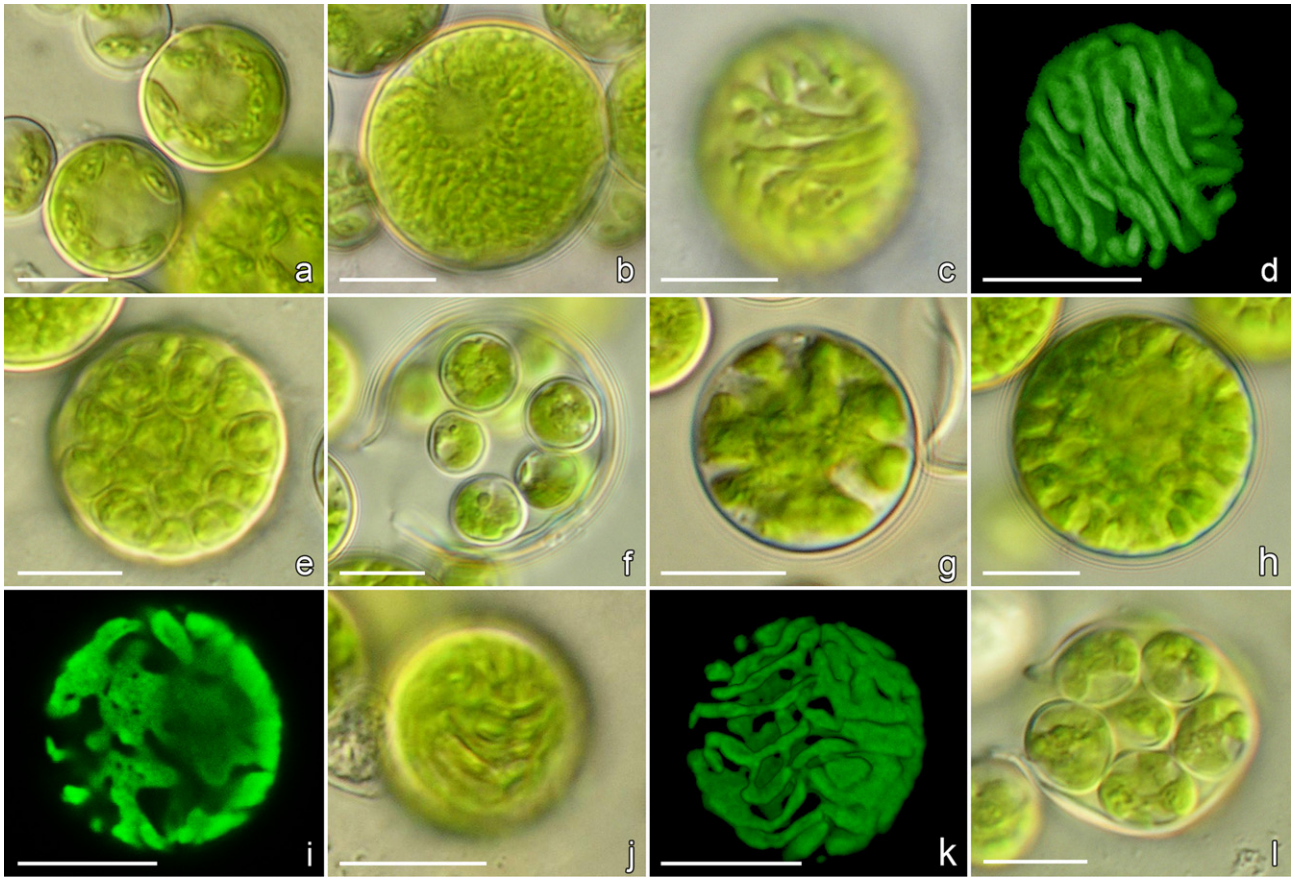

FIG. 4. Micrographs of investigated strains. (a–f) *Dictyochloropsis splendida* (CAUP H 8601, SAG 2071, SAG 2097). (a) Young cells. (b) Mature cell in optical section. (c) Chloroplast surface consisting of many parallel-arranged lobes. (d) Chloroplast structure of mature cell; chloroplast reconstruction made from multiple serial confocal sections. (e) Young autosporangium. (f) Opened mature autosporangium. (g–m) *Dictyochloropsis asterochloroides* (SAG 2073, SAG 2098). (g) Young cell with asteroid chloroplast. (h) Mature cell with many fine chloroplast lobes. (i) Chloroplast section showing thin tubular apertures; single median confocal section. (j) Parallel chloroplast lobes, surface view. (k) Chloroplast structure of mature cell; chloroplast reconstruction made from multiple serial confocal sections. (l) Opened mature autosporangium, scale bars = 10 μm.

autospores (16–32 daughter cells), a characteristic feature of the *Dictyochloropsis* clade 1, was extremely rare or absent in some strains. Therefore, differences seen in chloroplast morphology and asexual reproduction were congruent with the separation of the studied strains into two clades in the molecular phylogeny.

Lineages within *Dictyochloropsis* clade 2 are characterized by young cells with single parietal chloroplasts that have different patterns of reticulation. With maturity, many of the chloroplasts form lobes that penetrated into the cytoplasm, sometimes as far as the nucleus and become multilayered. Combinations of features observed in the chloroplast, asexual reproduction and cell size and shape clearly defined several groups of strains that corresponded to lineages within *Dictyochloropsis* clade 2 seen in the phylogeny (Fig. 3). For details, see Table 2.

Strains UTEX 2599 and UTEX 2612, previously assigned to *D. splendida*, shared identical morphology with strain SAG 12.86, the authentic strain of *D. symbiontica* var. *pauciautosporica* (Fig. 5, a–g). Vegetative cells of these strains were distinct by having a parietal chloroplast with almost the whole lumen filled by cytoplasm, making the centrally located nucleus clearly visible (Fig. 5e). In addition, chloroplast lobes were broader (~2 μm) than in any other investigated strain. With vegetative cells up to 30 μm in diameter these three strains were the largest in *Dictyochloropsis* clade 2.

The morphology of strains SAG 2036 and SAG 2154 (Fig. 5, h–l) clearly corresponded to the description of *D. irregularis* (Nakano and Isagi 1987, Tschermak-Woess 2000). The most distinct morphological feature was the variable shape of mature cells (Fig. 5, h–i). The sporangia formed a characteristic thickening of the inner cell wall at the broader pole of the cell (Fig. 5l).

Strain SAG 46.85 formed spherical cells with a predominantly multilayered chloroplast (Fig. 5, m–p). In surface view, the chloroplast had rather spherical round spaces between the lobes (Fig. 5o). Despite extensive investigation, the production of zoospores was not observed.

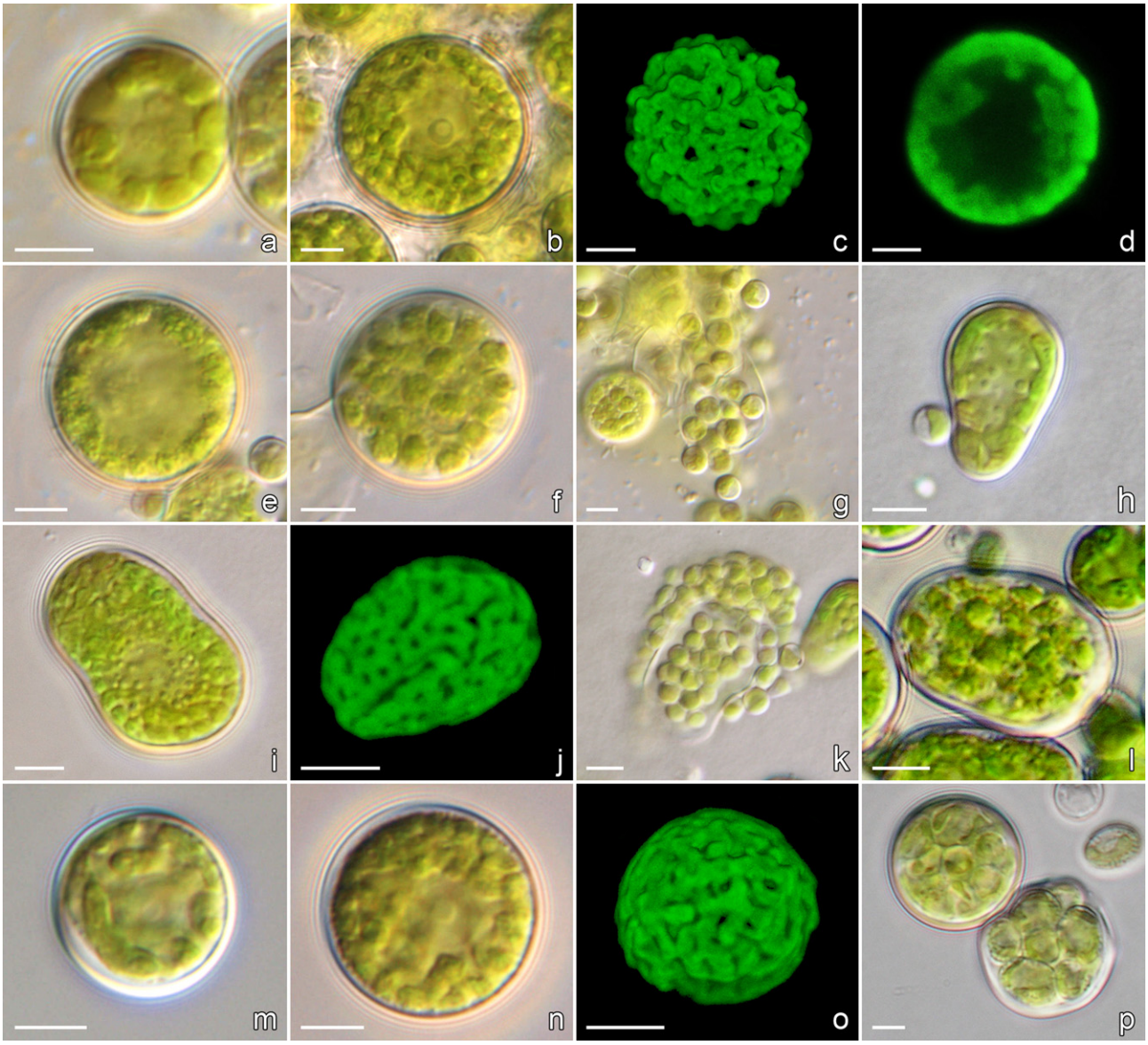

FIG. 5. Micrographs of *Symbiochloris*. (a–g) *Symbiochloris pauciautosporica* (SAG 12.86). (a) Young cell with parietal chloroplast. (b) Mature cell with multilayered chloroplast. (c) Chloroplast structure of mature cells, maximum projection of multiple serial confocal sections. (d) Chloroplast structure of mature cells, single median confocal section. (e) Mature cell with parietal chloroplast and a high portion of cell lumen filled by cytoplasm and centrally located nucleus. (f) Young aplanosporangium. (g) Mature opened aplanosporangium. (h–l) *Symbiochloris irregularis* (SAG 2154). (h) Young cell. (i) Mature cell with multilayered chloroplast. (j) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (k) Opened mature aplanosporangium. (l) Young aplanosporangium showing inner thickening of the cell wall. (m–p) *Symbiochloris tschermakiae* (SAG 46.85). (m) Young cell with newly formed chloroplast lobes. (n) Mature cell with multilayered chloroplast. (o) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (p) Mature autosporangium (bottom right) and aplanosporangium (top left). Note the irregular shape of mature autosporangium, assuming the shape of inlying autospores, scale bars = 5 μm.

The lineage formed by the authentic strain of *D. symbiontica*, SAG 27.81, together with Japanese strains SAG 2070 and SAG 2099, was characterized by spherical cells containing either parietal or multilayered chloroplasts (Fig. 6, a–k). In surface view, the chloroplast had many small, irregular spaces between the lobes (Fig. 6d). Asexual reproduction of the three strains was by means of 4–16 autospores (Fig. 6, e and f), released either by dissolving or rupturing of the sporangial cell wall. When dissolving, the autospores remained attached to each other forming packets of cells (Fig. 6f). Upon rupture, the autospores were not released, but stayed attached to the parent cell wall, even in widely opened sporangia (Fig. 6g). Sometimes colony-like packets of cells adhering to each other by sporangial wall remnants were observed (Fig. 6h). Often remnants of sporangial walls, resembling scales, were present on the surface of mature autospores and vegetative cells (Fig. 6i).

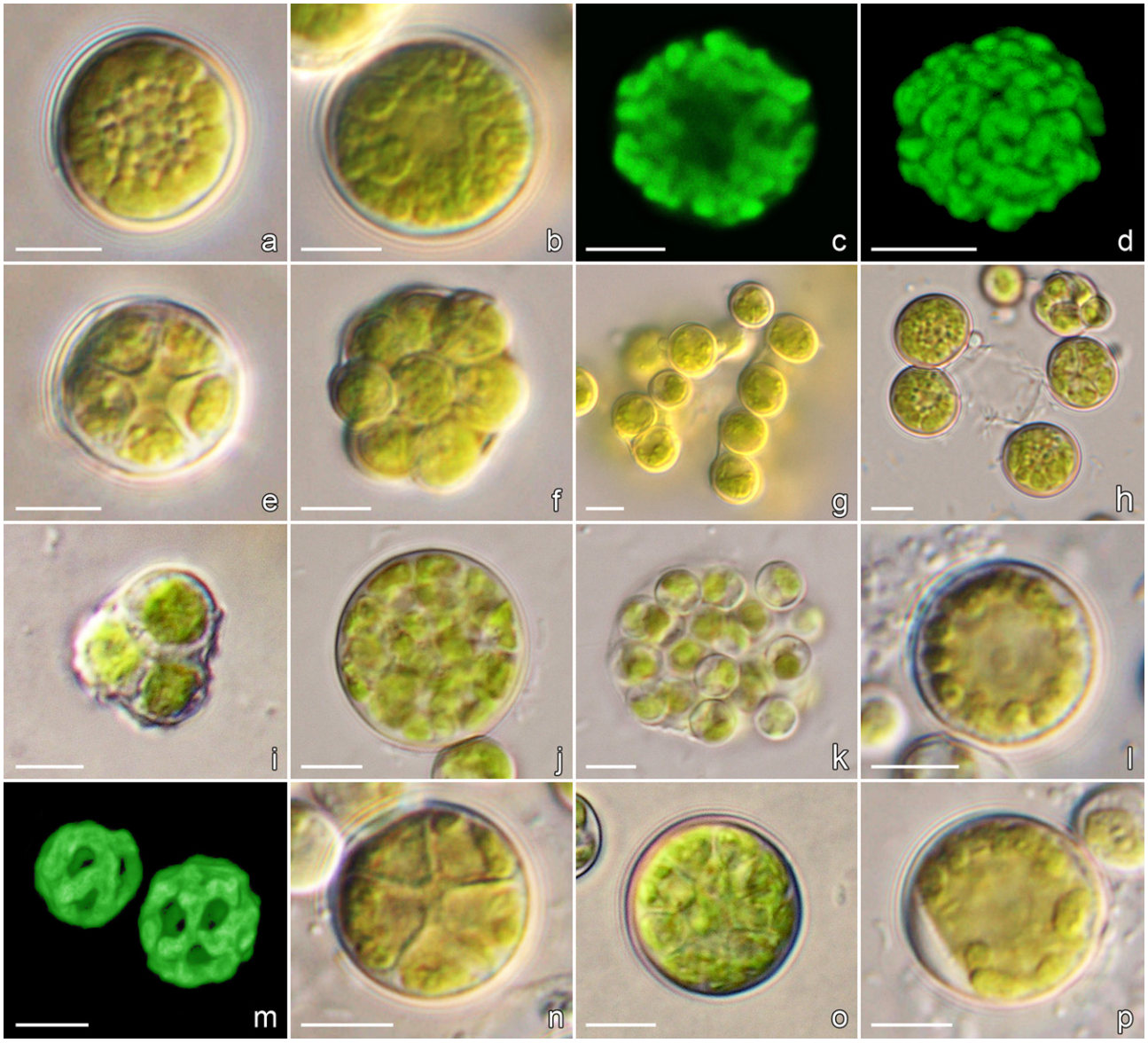

FIG. 6. Micrographs of *Symbiochloris*. (a–k) *Symbiochloris symbiontica* (SAG 27.81, SAG 2070). (a) Young cell. (b) Mature cell with multilayered chloroplast. (c) Multilayered chloroplast in mature cell, single median confocal section. (d) Chloroplast structure of mature cell, maximum projection of multiple serial confocal sections. (e) Young autosporangium. (f) Mature autosporangium with dissolved mother cell wall. (g) Chain of autospores adhered to the mother cell wall. (h) Packet of four cells adhered each to other by mother sporangium cell wall remnants. (i) Mature autospores with scales sticked on the cell surfaces. (j). Young aplanosporangium. (k) Opened mature aplanosporangium. (l–p) *Symbiochloris reticulata* (SAG 53.87). (l) Mature cell with a distinct nucleus. (m) Chloroplast structure of mature cells, maximum projection of multiple serial confocal sections. Note large chloroplast pores (n) Young autosporangium. (o) Mature aplanosporangium. (p) Young aplanosporangium showing inner thickening of the cell wall, scale bars = 5 μm.

Strain SAG 53.87, assigned to *D. reticulata*, was characterized by a parietal chloroplast with interconnected lobes even in mature cells (Fig. 6l), with large distinct spaces between the lobes (Fig. 6m). Asexual reproduction was by means of 4–8(-16) autospores (Fig. 6n), similar to *D. symbiontica* strains. In contrast to the latter strains, in strain SAG 53.87 autospores were released by rupture and did not adhere to each other by remnants of the sporangial wall. Despite extensive investigation, the production of zoospores was not observed in this strain, and aplanospores (16–32 per sporangium) were formed only rarely (Fig. 6o). However, we occasionally noticed inner thickenings of the sporangial cell wall that was found in early stages of aplanospore and zoospore formation of other *Dictyochloropsis* clade 2 strains (Fig. 6p).

Two strains, SAG 2150 and CAUP H 8602, differed from the rest by the occasional production of ellipsoidal cells. The chloroplast of strain SAG 2150, even in many mature cells, was parietal and reticulate, with the cell lumen filled by cytoplasm and

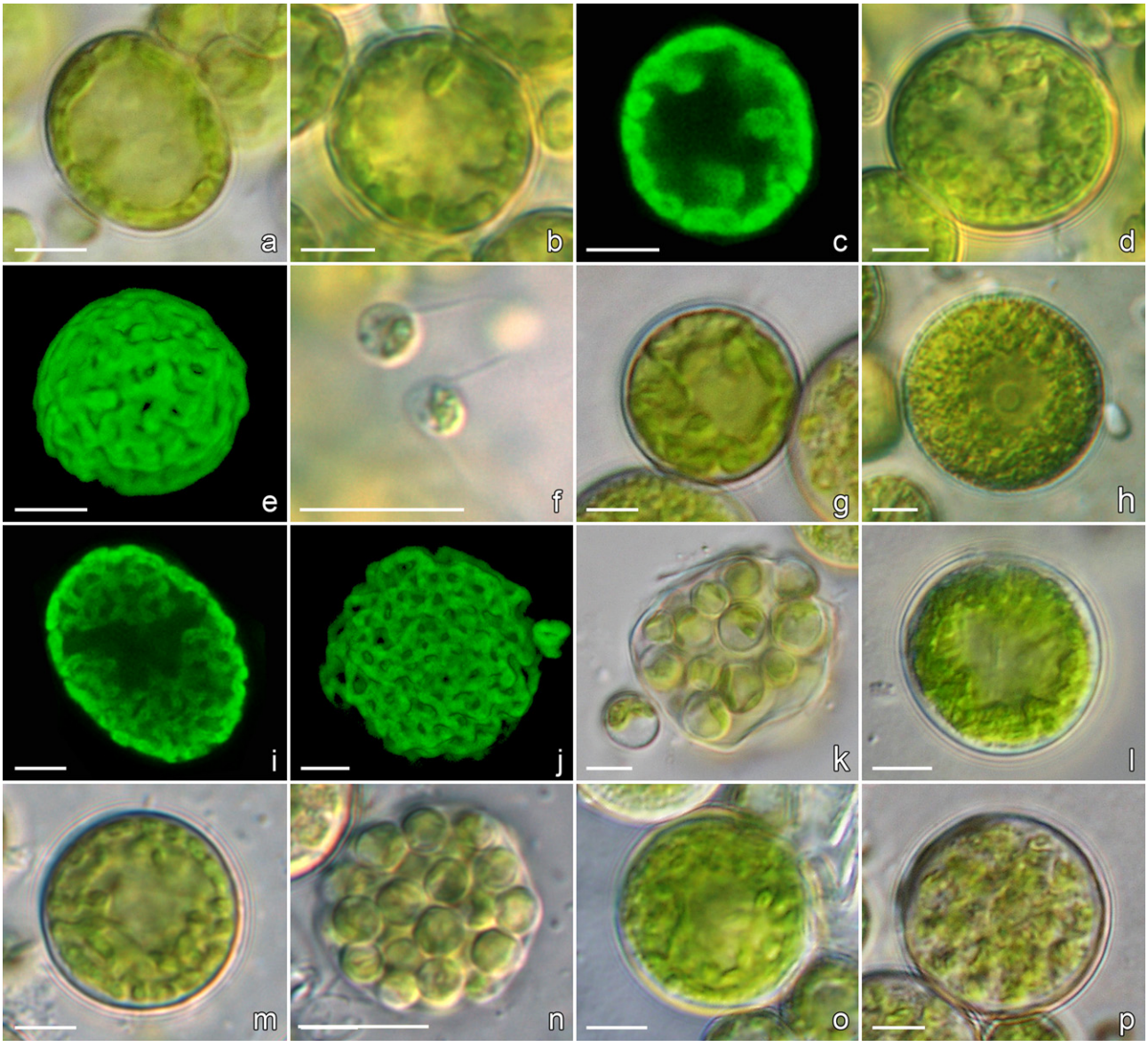

FIG. 7. Micrographs of *Symbiochloris*. (a–f) *Symbiochloris handae* (SAG 2150). (a) Young cell with parietal chloroplast. (b) Mature cell with a few solitary lobes spreading the cell centre. (c) Chloroplast section showing solitary lobes spreading the cell centre, single median confocal section. (d) Mature cell with multilayered chloroplast. (e) Chloroplast structure of mature cell, 3D reconstruction. (f) Zoospores with subapical isokont flagella emerging symmetrically near the flattened apex. (g–k) *Symbiochloris tropica* (CAUP H 8602). (g) Young cell. (h) Mature cells with multilayered chloroplast. (i) Chloroplast structure of mature cells, single median confocal section. (j) Chloroplast structure of mature cells, 3D reconstruction. (k) Opened mature aplanosporangium. (l) *Symbiochloris* sp. SAG 2069; mature cell with parietal chloroplast. (m–n) *Symbiochloris* sp. SAG 244.80. (m) Mature cell. (n) Mature aplanospores. (o–p) *Symbiochloris* sp. CAUP H 8603. (o) Mature cells with multilayered chloroplast. (p) Young aplanosporangium, scale bars = 5 μm.

one distinct nucleus (Fig. 7a). However, in several mature cells, new chloroplast lobes, oriented perpendicular to the parietal chloroplast and penetrating into the cell, arose from the parietal chloroplast (Fig. 7, b and c). Rarely, the chloroplast developed into the multilayered stage in mature cells (Fig. 7d), but, when present, differed in shape from all other investigated strains by having a sparser net of interconnected lobes (compare Fig. 7d with 5b and 6b). Moreover, in surface view, the chloroplast had small linear fissures (Fig. 7e), rather than distinct spherical pores. Mature autospores and vegetative cells often remained attached each other forming very large cell packets. Usually 64–128 zoospores (Fig. 7f) or aplanospores were formed in globular or slightly ellipsoidal sporangia.

The chloroplast of strain CAUP H8602 was predominantly multilayered (Fig. 7, g–j). In contrast to other strains, the chloroplast did not possess a granulate structure, but was formed by many thin tubular lobes (compare Fig. 7h with 5, b and n). In surface view, the chloroplast had rather small spaces

TABLE 2. Morphological characterization of investigated *Dictyochloropsis* and *Symbiochloris* species.

Species	Vegetative cells		Chloroplast morphology				Reproduction				
	Cell shape	Cell diameter (μm)	Parallel-oriented chloroplast lobes	Multilayered chloroplast	Asteroid chloroplast	Chloroplast pores in surface view	Number of autospores	Number of aplanospores	Number of zoospores	Sporangium cell wall development	Autospores forming chains
D. splendida	Spherical	6–30 (–40)	+	+	–	Absent	(4-)8–16	Not observed	Not observed	Rupturing	–
D. asterochloroides	Mostly spherical	6–22 (–24)	+	+	+	Absent	4–8	Not observed	Not observed	Rupturing	–
S. symbiontica	Mostly spherical	4.5–16 (–18)	–	+	–	Small, irregular	4–16	16–32	32	Rupturing or dissolving	+
S. reticulata	Mostly spherical	4–13 (–15)	–	–	–	Large, irregular	4–16	16–32	Not observed	Rupturing or dissolving	–
S. pauciautosporica	Mostly spherical	3.5–28 (–30)	–	+	–	Small, spherical	Not observed	64–128	64	Rupturing	–
S. irregularis	Variable in shape	3.5–20 (–22)	–	+	–	Small, spherical	Not observed	32–128	64–128	Rupturing	–
S. tschermakiae	Mostly spherical	5–16 (–19)	–	+	–	Large, spherical	4–16	32	Not observed	Rupturing or dissolving	–
S. handae	Spherical, ellipsoidal	3.5–26 (–30)	–	+	–	Small, linear	8–32	64–128	64	Rupturing or dissolving	–
S. tropica	Spherical, ellipsoidal	3–24 (–26)	–	+	–	Small, spherical	Not observed	32–64	32–64	Rupturing	–

between the lobes (Fig. 7j). Reproduction was by means of 16–64 aplanospores and zoospores, released by the rupturing of sporangia (Fig. 7k) so the remnants of sporangial cell walls remained in the culture.

For SAG 244.80, SAG 2069, and CAUP H8603 strains belonging to *Dictyochloropsis* clade 2, we found virtually no distinct morphological features to clearly delimit them from other species. Strains SAG 244.80 and SAG 2069 were morphologically similar to *D. symbiontica* var. *pauciautosporica* (SAG 12.86). Both strains shared vegetative cells 20(-23) μm in diameter with a parietal chloroplast and one distinct nucleus (Fig. 7, l and m), asexual reproduction by 64–128 aplanospores or zoospores (Fig. 7n), and no autospores. Strain CAUP H8603 had no specific morphological diagnostic features. Cells were spherical, up to 21(-24) μm in diameter. In surface view, the chloroplast had rather round spaces between the lobes (Fig. 7o). Asexual reproduction was by means of 32–64 zoospores or aplanospores that were released by either dissolving or rupturing of the sporangial wall (Fig. 7p). Autospore production was not observed.

Molecular species identification. On the basis of phylogenetic evidence, we tested a 3-species hypothesis for *Dictyochloropsis* clade 1 and a 12-species hypothesis for *Dictyochloropsis* clade 2 using BP&P and Stacey for species delimitation. For *Dictyochloropsis* clade 1, BP&P supported a two-species hypothesis, whereas Stacey supported the split of *D. splendida* into two species (Fig. 2). As we did not find any morphological evidence to support the split, we followed a more conservative species delimitation approach and considered strains of *D. splendida* to be one species.

Both analyses supported the hypothesis of 12 species in *Dictyochloropsis* clade 2 (Fig. 3 and Table 3), and the proposed species delineation was congruent with morphological data. Seven of the 12 lineages proposed to represent different species can be differentiated by the combined morphological features of chloroplast morphology, cell size, and mode of reproduction (Table 2).

Differences in the ITS secondary structure support the 12 species hypothesis of *Dictyochloropsis* clade 2 (Fig. S1 in the Supporting Information)—often involving correlated base changes in conserved regions. These highlight the genetic diversity between the species.

DISCUSSION

Taxonomic revision and taxa descriptions. Geitler (1966) described *Dictyochloropsis* with *D. splendida* as type species of the genus and noted that autosporogenesis was the only type of asexual reproduction. The production of wall-less zoospores was later mentioned by Tschermak-Woess, who described several new species of *Dictyochloropsis* and emended Geitler's

TABLE 3. Posterior probability of each delimited *Dictyochloropsis* and *Symbiochloris* species calculated by BP&P.

Code	Species	Posterior probabilities
Dictyochloropsis		
SAG 2097	*D. splendida*	0.53
SAG 2305		
CAUP H 8601		
SAG 2071		
SAG 2098	*D. asterochloroides*	0.98
SAG 2073		
Symbiochloris		
SAG 2150	*S. handae*	0.80
SAG 2036	*S. irregularis*	0.86
SAG 2154		
SAG 12.86	*S. pauciautosporica*	0.90
UTEX 2599		
MPN167		
MPN124		
SAG 53.87	*S. reticulata*	1.00
SCH2339		
SCH1069		
AB06006A2		
SCH2021		
SAG 27.81	*S. symbiontica*	0.90
SAG 2070		
SAG 2099		
CAUP H 8602	*S. tropica*	0.80
SAG 46.85	*S. tschermakiae*	1.00
SCH6004		
SCH12317		
SCH17084		
MPN169		
SA5417	*S.* S3	0.99
MPN168		
SCH6057		
SCH6058		
SA5523		
SA5534		
SAG 244.80	*S.* S6	0.80
SCH17733	*S.* S7	0.98
SCH1998		
SAG 2069	*S.* S8	0.80
CAUP H 8603	*S.* S11	0.81

description of the genus (Tschermak-Woess 1980, 1984). Since no authentic strain of the type species *D. splendida* was retained, she modified the genus description based on observations of the strain SAG 244.80 that was isolated from the lichen *Chaenotheca brunneola* (Tschermak-Woess 1978a). This strain was characterized by frequent zoospore production. However, the analyses of 18S and *rbc*L sequences clearly demonstrated the unrelated position of SAG 244.80 strain to all autosporine *D. splendida* strains, thus the emendation of the genus *Dictyochloropsis* that was made by Tschermak-Woess (1978a) has to be rejected.

Dictyochloropsis is polyphyletic, and two genera need to be recognized as inferred from ML and Bayesian analyses of 18S rRNA gene sequences and as supported by differences in chloroplast structure of mature cells and the mode of asexual reproduction (Table 2). Following the original definition of Geitler (1966), we propose here to restrict *Dictyochloropsis* to vegetative unicells whose chloroplast surface at some ontogenetic stage has distinct parallel-arranged lobes and whose reproduction is by asexual autospores. *Dictyochloropsis* therefore includes all free-living strains of *Dictyochloropsis* clade 1 sensu Dal Grande et al. (2014). We also propose a new genus, *Symbiochloris*, for all free-living and/or lichenized strains in *Dictyochloropsis* clade 2 sensu Dal Grande et al. (2014) characterized by vegetative unicells having a parietal, reticulate chloroplast with lobes not in a parallel arrangement and reproducing asexually by aplanospores and/or by naked biflagellate zoospores with widely separated, subapical isokont flagella (Fig. 7f). *Dictyochloropsis* sensu Tschermak-Woess comprised seven taxa (Index Nominum Algarum 2015): *D. irregularis*, *D. reticulata*, *D. splendida*, *D. splendida* var. *gelatinosa* Tschermak-Woess, *D. symbiontica*, *D. symbiontica* var. *ellipsoidea* Tschermak-Woess, and *D. symbiontica* var. *pauciautosporica*. With the exception of the type species, *D. splendida*, descriptions of the chloroplast morphology given in the diagnoses of these taxa clearly indicate that they belong to the newly proposed genus *Symbiochloris*.

The use of a multiphasic approach enabled us to investigate species-level diversity in both genera. By applying a species concept based on a combination of morphological features such as chloroplast shape, sporogenesis, cell shape, cell dimensions, as well as DNA sequences, we delimited two species in the *Dictyochloropsis*, and 12 species in *Symbiochloris*.

Dictyochloropsis Geitler 1966: 162

Type species: *Dictyochloropsis splendida* Geitler

Description: Cells spherical, uninucleate, with smooth, thin cell wall. Chloroplast without pyrenoid, reticulate, complex in structure and filling entire mature cell except area occupied by central nucleus; chloroplast of many interconnected lamellae that often appear parallel at its periphery. Asexual reproduction by spherical autospores, no zoospores or aplanospores produced. Autosporangia spherical, at maturity assume shape of inlying autospores. Daughter cells released by rupturing of sporangium cell wall.

Dictyochloropsis splendida Geitler 1966:162–163, Figure 1.

Description: Cells solitary, spherical, up to 20(–30) μm diameter. Chloroplast in young cells single, parietal, with network of lobes below cell membrane; in mature cells, forming complex net of interconnected lobes. Reproduction exclusively by (4-)8–16 autospores each ~8–15 μm in diameter, liberated by rupturing of parent cell wall. Diagnostic DNA sequences: 18S: GU017662, *rbc*L: KC333607, ITS: KC333515.

Lectotypus (here designated): Geitler 1966, *Österr. Bot. Z.* 133:162–163, Figure 1.

Epitypus (here designated): Strain CAUP H8601 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the

Culture Collection of Algae of Charles University in Prague (CAUP), Benátská 2, Praha 2, Czech Republic.

Type Locality: soil sample, ventarole, top of the Boreč hill, České Středohoří Mts., Czech Republic. Sample collected by Pavel Škaloud on February 8, 2003.

Habitat: Aero-terrestrial; on surface of bark (SAG 2305), and artificial substrata (e.g., concrete; SAG 2071, SAG 2097), and in soil (CAUP H8601).

Remarks: Presently, several isolates of *D. splendida* are available in culture collections and could possibly represent the organism to which the name is linked. An isolate of *D. splendida* from a lichen thallus was identified by Tschermak-Woess (1978a,b) and deposited as strain SAG 244.80. Later, two strains of *D. splendida* isolated from the lichen *Phlyctis argena* were deposited as strains UTEX 2599 and UTEX 2612 by Tschermak-Woess (1995). An isolate from soil substrate has been identified by Škaloud et al. (2005) as *D. splendida* and deposited as strain CAUP H8601 (Table 1). Our observations on the morphology of vegetative cells and on asexual reproduction of strains SAG 244.80, UTEX 2599 and UTEX 2612 do not agree with Geitler's (1966) original description of *D. splendida*. On the contrary, cell dimensions, the parallel-arrangement of the chloroplast lobes, and asexual reproduction by autospores observed in strain CAUP H8601 are all in agreement with the original description of *D. splendida*. We therefore proposed strain CAUP H8601 as the epitype of *D. splendida*. The morphology and chloroplast ontogeny of the epitype was studied in detail by Škaloud et al. (2005).

Dictyochloropsis asterochloroides Škaloud, Friedl, A.Beck & Dal Grande **sp. nov.** (Fig. 4, g–m).

Description: Cells solitary, spherical, or slightly ellipsoidal, 6–22(–24) μm diameter with single parietal or central and stellate chloroplast. Nucleus very indistinct, parietal, with nucleolus. Chloroplast, in some portion of mature cells, remains central with few thick or many thinner lobes spreading out to cell periphery; chloroplast, in rest of cells, complex, of many interconnected, evenly distributed lobes, with central nucleus. Chloroplast lobes appearing flat and parallel often produced. By confocal microscopy, chloroplast with thin tubular apertures. Reproduction by 4–8 autospores, each about 6 μm diameter, liberated by rupturing of parent cell wall. Diagnostic DNA sequences: 18S: GU017664, *rbc*L: KC333606.

Holotypus: Strain SAG 2098 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany. Living cultures have been deposited as SAG 2098 and SAG 2073 and as CAUP H8604 in CAUP, Benátská 2, Praha 2, Czech Republic.

Etymology: The specific epithet refers to the central and stellate shape of chloroplast in young cells (Fig. 4g).

Type locality: Yokogawa, Hiroshima-City, Japan, on concrete. Sample collected by Shinji Handa in May, 2004.

Habitat: Aero-terrestrial; on artificial substrata (e.g., concrete).

Symbiochloris Škaloud, Friedl, A.Beck & Dal Grande **gen. nov.**

Synonyms: *Dictyochloropsis* Geitler emend. Tschermak-Woess 1984, *Pl. Syst. Evol.* 147, p. 317, non *Dictyochloropsis* Geitler 1966, *Osterr. Bot. Z.* 133, p. 162.

Description: Cells spherical, ellipsoidal, or irregularly shaped, with smooth, thin, unornamented cell wall, each with one central nucleus with prominent nucleolus. Chloroplast shape varies with cell age; in young cells, single and parietal of interconnected lobes; in mature cells, chloroplast may develop multilayered reticulum of lobes (never arranged in parallel) filling cell; in surface view appears reticulate. Pyrenoid absent. Asexual reproduction by zoospores and two types of immobile daughter cells—aplanospores and autospores. Young aplanosporangia characterized by indistinct cell content, starch accumulation and yellowish or pale green color; mature aplanosporangia spherical or ellipsoidal, with numerous (up to 128 cells), spherical, daughter cells released by rupturing or dissolving of sporangial cell wall. Autosporangia, if present, characterized by distinct, angular cell walls of new produced cells, no starch accumulation and dark green color; when mature assuming shape of inlying autospores; mature autospores deformed by close contact of daughter cells inside sporangium, not strictly spherical when released; commonly, 4–16 autospores produced within sporangium, released by rupturing or dissolving of sporangial cell wall. Zoospores produced in spherical or ellipsoidal zoosporangia, having same characteristics as aplanosporangia. Early stages of zoosporangia development characterized by inner cell wall thickening on one side of cell. Zoospores released by sporangial cell wall rupture, developed in position of earlier thickening. Biflagellate zoospores naked, with subapical isokont flagella emerging symmetrically near the flattened apex. They are ellipsoidal or cylindrical, with one parietal chloroplast without an observable stigma.

Etymology: The name refers to the common occurrence of the genus as a lichen photobiont.

Type species: ***Symbiochloris pauciautosporica*** (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande **stat. et comb. nov.** (Fig. 5, a–g)

Basionym: *Dictyochloropsis symbiontica* var. *pauciautosporica* Tschermak-Woess 1984, *Pl. Syst. Evol.* 147, p. 317, Figs. 6 and 7.

Holotypus: A lichen *Pseudocyphellaria aurata*, collected by Metlesics (14. 5.1983), deposited in the

herbarium of the Institute of Botany, University of Vienna, Austria (WU).

Epitypus (here designated): Strain SAG 12.86 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany.

Type locality: Tenerife (Spain), Anaga-Mts., Pico de Chinobre, 600–800 m a.s.l.

Emended diagnosis: Cells spherical, 3.5–28(-30) µm diameter. Chloroplast as for genus; in some mature cells remain parietal. Asexual reproduction by means of 64–128 aplanospores and 64 zoospores, released by rupturing of sporangial cell wall. Diagnostic DNA sequence: 18S: GU017651.

Habitat: Photobiont of lichens *Phlyctis argena* and *Crocodia aurata* (Tschermak-Woess 1984, 1995).

Investigated strains: SAG 12.86 (authentic strain), UTEX 2599, UTEX 2612.

Symbiochloris irregularis (Tak.Nakano & Isagi) Škaloud, Friedl, A.Beck & Dal Grande **comb. nov.** (Fig. 5, h–l)

Basionym: *Dictyochloropsis irregularis* Tak.Nakano and Isagi 1987, *Phycologia* 26, p. 224, figures 1–16.

Holotypus: Nakano and Isagi (1987), Figure 4.

Epitypus (here designated): Strain SAG 2036 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany.

Emended diagnosis: Cells ellipsoidal, ovoid, pyriform, reniform or irregularly oblong, up to 25 µm long and 18 µm wide. Chloroplast as for genus; has rather round spaces between lobes. Asexual reproduction by means of 32–128 aplanospores, and 64–128 zoospores formed in sporangia of pyriform or reniform shape. Mature spores released by rupturing of sporangial cell wall. Diagnostic DNA sequences: 18S: GU017659, *rbc*L: KC333600.

Habitat: Free-living, subaerial on the bark of *Picea abies* and *P. jezoensis* (Nakano and Isagi 1987, Tschermak-Woess 2000).

Investigated strains: SAG 2036, SAG 2154.

Symbiochloris tschermakiae Škaloud, Friedl, A.Beck & Dal Grande **sp. nov.** (Fig. 5, m–p)

Description: Cells solitary, spherical, or slightly ellipsoidal. Cells 5–16(-19) µm diameter. Asexual reproduction by means of autospores, aplanospores, and zoospores. Autosporangia to 13 µm in diameter, containing 4–16 autospores released by either dissolving or rupturing of sporangial cell wall. After rupturing of sporangium, autospores remain attached to parent cell wall, forming chains of cells or cell packets. Aplanosporangia 16–18 µm in diameter, containing 32 spores released by rupturing of sporangial cell wall. Zoospores not observed. Diagnostic DNA sequences: 18S: GU017654, *rbc*L: KC333574, ITS: KC333521.

Holotypus: Strain SAG 46.85 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany. Living cultures have been deposited as SAG 46.85 and as CAUP H8605 in CAUP, Benátská 2, Praha 2, Czech Republic.

Etymology: The specific epithet is chosen in honor of Dr. Elisabeth Tschermak-Woess, who described the majority of *Dictyochloropsis* taxa.

Type locality: North Auckland, North Island, New Zealand. Sample was collected by Elisabeth Tschermak-Woess in 1981.

Habitat: Widespread photobiont of *Crocodia aurata*, also found in *Lobaria patinifera*.

Symbiochloris symbiontica (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande **comb. nov.** (Fig. 6, a–k)

Basionym: *Dictyochloropsis symbiontica* Tschermak-Woess 1980, *Pl. Syst. Evol.* 136, pp. 304–305, figures 5–7.

Holotypus: A lichen, *Chaenothecopsis consociata*, collected by Tschermak-Woess (1.11.1976), deposited in the herbarium of the Institute of Botany, University of Vienna, Austria (WU).

Epitypus (here designated): Strain SAG 27.81 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany.

Type locality: Lunz (Austria). Sample collected by Elisabeth Tschermak-Woess on November 1st, 1976.

Emended diagnosis: Cells spherical to slightly ellipsoidal, 4.5–16(-18) µm diameter. Asexual reproduction by means of 4–16 autospores, rarely by 16–32 zoospores and aplanospores. After rupturing of sporangium, autospores remain attached to parent cell wall, forming chains of cells or cell packets. Remnants of sporangial cell wall often present on surface of mature autospores and vegetative cells, resembling scales. Diagnostic DNA sequences: 18S: GU017644, ITS: KC333512.

Habitat: Photobiont of lichen *Chaenothecopsis consociata* (Tschermak-Woess 1980; SAG 27.81), and free-living on the surface of a lichen, *Graphis* sp. (SAG 2070, SAG 2099).

Symbiochloris reticulata (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande **comb. nov.** (Fig. 6, l–p)

Basionym: *Myrmecia reticulata* Tschermak-Woess 1969, Österr. Bot. Z. 98, pp. 412–419.

Synonym: *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess 1984, *Pl. Syst. Evol.* 147, p. 317.

Lectotypus (here designated): Tschermak-Woess (1951), Figure 1.

Epitypus (here designated): Strain SAG 53.87 permanently cryopreserved in a metabolic inactive

state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany.

Type locality: Tenerife (Spain), Roque Chinobre. Sample was collected by Elisabeth Tschermak-Woess in 1978.

Emended diagnosis: Cells spherical or slightly ellipsoidal, up to 4–13(-15) μm in diameter. Chloroplast strictly parietal, with granulate structure, suggestive of many small tightly appressed spherical chloroplasts. Asexual reproduction by 4–8(-16) autospores, released by rupturing of sporangial cell wall. Diagnostic DNA sequences: 18S: GU017650, *rbc*L: KF960688, ITS: KC333513.

Habitat: Widespread photobiont of Lobariaceae, namely *Lobaria pulmonaria* (Tschermak-Woess 1978b), *L. crassior*, *L. gyrophorica*, *L. immixta*, *L. isidiophora*, *L. japonica*, *L. macaronesica*, *L. orientalis*, *L. pindarensis*, *L. sachalinensis*, *L. spathulata*, *L. sublaevis*, *L. tuberculata*, *L. virens*, *L. yunnanensis*, *Dendriscosticta platyphylloides*, *D. praetextata*, *D. wrightii*, *Ricasolia amplissima* (Dal Grande et al. 2014).

Remarks: For the epitype, we chose strain SAG 53.87, isolated from lichen *Lobaria pulmonaria* by Tschermak-Woess (1978b). The morphology and ecology of this strain (lichen photobiont) clearly corresponded to the original description of the basionym, *M. reticulata* (Tschermak-Woess 1951). Moreover, the strain used for the epitypification was isolated and determined by the same author who originally described the basionym.

Symbiochloris handae Škaloud, Friedl, A.Beck & Dal Grande **sp. nov.** (Fig. 7, a–f)

Description: Cells solitary, spherical, or ellipsoidal. Chloroplast as for genus; in surface view, has small linear spaces between the lobes. Cell diameters 3.5–26(-30) μm. Asexual reproduction by means of autospores, aplanospores, and zoospores. Autosporangia ellipsoidal or spherical, up to 22 μm in diameter, containing 8–32 autospores released by dissolving of sporangial cell wall. Mature autospores and vegetative cells often remain attached each other forming very large cell packets. Zoosporangia and aplanosporangia globular or slightly ellipsoidal up to 22 μm in diameter, containing 64–128 aplanospores or 64 zoospores. Zoospores released by rupturing of sporangial cell wall, autospores released by cell wall rupturing, followed by its dissolving. Zoospores ellipsoidal to cylindrical, without stigma, 5–8 μm in length and 3 μm in width, flagella as for genus. Diagnostic DNA sequences: 18S: Z47207, *rbc*L: KC333590, *ITS*: KC333524.

Holotypus: Strain SAG 2150 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in the Sammlung für Algenkulturen der Universität Göttingen (SAG), Nikolausberger Weg 18, Göttingen, Germany. The living cultures have been deposited as SAG 2150 and as CAUP H8606 in CAUP, Benátská 2, Praha 2, Czech Republic.

Etymology: The specific epithet honors Dr. Shinji Handa, who collected most of the strains investigated in this study.

Type locality: near Hiroshima, Japan. Sample was collected by Shin Takeshita.

Habitat: Phycobiont of lichen *Brigantiaea ferruginea*.

Investigated strain: SAG 2150.

Symbiochloris tropica Škaloud, Friedl, A.Beck & Dal Grande **sp. nov.** (Fig. 7, g–k)

Description: Cells solitary, spherical or ellipsoidal. Chloroplast as for genus; mature, multilayered chloroplast formed by many thin tubular lobes; in surface view with small round spaces between lobes. Cells 3–24(-26) μm diameter. Asexual reproduction by means of 32–64 aplanospores and zoospores, formed in globular or slightly ellipsoidal sporangia up to 25 μm diameter. Spores released by rupturing of sporangia. Zoospores ellipsoidal, without stigma, 5.5–6 μm long and 2–3 μm wide, flagella as for genus. Autospores not observed. Diagnostic DNA sequences: 18S: GU017655, *rbc*L: KC333603, ITS: KC333516.

Holotypus: Strain CAUP H 8602 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) in CAUP, Benátská 2, Praha 2, Czech Republic. The living culture has been deposited as CAUP H 8602.

Etymology: The specific epithet refers to the tropical origin of the isolated strain.

Type locality: Bark sample, secondary lowland rain forest, Hulu Kelantan, Malaysia.

Investigated strain: CAUP H 8602.

Symbiochloris gelatinosa (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande **stat. et comb. nov.**

Basionym: *Dictyochloropsis splendida* var. *gelatinosa* Tschermak-Woess 1984, *Pl. Syst. Evol.* 147, pp. 316–317, figures 1–3.

Holotypus: A lichen *Catinaria grossa*, collected by Sipman (May 14, 1983 collection #16778), deposited in the herbarium of the Institute of Botany, University of Vienna, Austria (WU).

Habitat: Photobiont of lichen *Catinaria grossa* (Tschermak-Woess 1984).

Remarks: This strain is characterized by the production of a thick mucilaginous envelope of cultured cells (Tschermak-Woess 1984).

Symbiochloris ellipsoidea (Tschermak-Woess) Škaloud, Friedl, A.Beck & Dal Grande **stat. et comb. nov.**

Basionym: *Dictyochloropsis symbiontica* var. *ellipsoidea* Tschermak-Woess 1980, *Pl. Syst. Evol.* 136, p. 305, figure 8.

Lectotypus (here designated): A lichen, *Chaenothecopsis consociata*, collected by Hafellner (October 10, 1976) deposited in the herbarium of the Karl Institute of Botany, Franzens University of Graz, Austria (GZU).

Syntypus (here designated): A lichen, *Chaenothecopsis consociata*, collected by Hafellner & Titze (July 29, 1978), deposited in the herbarium of the Institute of Botany, University of Vienna, Austria (WU).

Habitat: Photobiont of lichen *Chaenothecopsis consociata* (Tschermak-Woess 1980).

Remarks: This strain is characterized by the production of ellipsoidal cells, and of autospores that are often covered by remnants of sporangial walls, resembling scales (Tschermak-Woess 1980). Since two specimens were simultaneously designated as types of *D. symbiontica* var. *ellipsoidea*, we selected the older specimen as the lectotype.

Key to species of *Dictyochloropsis* and *Symbiochloris* (Fig. S2 in the Supporting Information).

1.	Chloroplast lobes in closely spaced parallel arrangement; reproduction strictly by autospores (*Dictyochloropsis*)	2
	Chloroplast lobes wrinkled and close together; reproduction by aplanospores, zoospores and autospores (*Symbiochloris*)	3
2.	Cells up to 50 μm in diameter; chloroplast of young cells parietal.	*D. splendida*
	Cells up to 24 μm in diameter; chloroplast of young cells asteroid.	*D. asterochloroides*
3.	Autosporogenesis (up to 32 spores) as prevailing type of asexual reproduction	4
	Aplanosporogenesis (up to 128 spores) as prevailing type of asexual reproduction	6
4.	Young autospores often remain attached to sporangial cell wall, forming chains of cells or cell packets	*S. symbiontica*
	Young autospores never remain attached to sporangial cell wall	5
5.	Chloroplast multilayered in mature cells	*S. tschermakiae*
	Chloroplast parietal in mature cells	*S. reticulata*
6.	Mature cells spherical or ellipsoidal	7
	Mature cells variable in shape	*S. irregularis*
7.	Cells often ellipsoidal	8
	Ellipsoidal cells never formed or very rare	10
8.	Chloroplast in mature cells always multilayered	9
	Chloroplast in mature cells often parietal, if multilayered, lobes sparsely interconnected, oriented perpendicularly to original layer	*S. handae*
9.	Autospores covered by remnants of sporangial walls resembling scales	*S. ellipsoidea*
	Scales not produced	*S. tropica*
10.	Cells covered by a thick mucilaginous envelope	*S. gelatinosa*
	Mucilaginous envelope not formed	*S. pauciautosporica*

Phylogeny and molecular diversity. Both *Dictyochloropsis* and *Symbiochloris* are positioned deep within the Trebouxiophycean lineage together with autospore (e.g., *Chlorella*, *Coccomyxa* Schmidle) and zoospore (e.g., *Trebouxia* de Puymaly, *Myrmecia* Printz) producing algae (Fig. 1). Thus, they probably evolved from a common ancestor that asexually reproduced by means of zoospores (Friedl 1995). Autosporine and zoosporine species are mixed throughout the entire phylogenetic tree of Trebouxiophyceae, and our phylogenetic analysis strongly supports the zoosporine genus *Symbiochloris* in a clade otherwise comprised of only autosporine species (i.e., *Viridiella* Albertano, Pollio & Taddei, *Chloroidium* Nadson, *Heveochlorella* Zhang, Huss, Sun, Chang & Pang, and *Watanabea* Hanagata, Karube, Chihara & P.C.Silva). The inferred tree topology suggests that the loss of zoosporogenesis may have occurred independently several times in the phylogenic history of Trebouxiophyceae (Friedl 1995) or that our knowledge of asexual reproduction in many genera is insufficient. However, the absence of zoospores in *Dictyochloropsis* is supported not only by our intensive morphological investigation of several strains, but also by the absence of aplanospores, a type of immotile asexual spore, ontogenetically affined to zoospores (Friedl 1993). As our results show, the species diversity of *Symbiochloris* is much higher than currently recognized and likely additional species will be described.

In addition, our findings do not support the hypothesis that lichenized algae evolve at a slower rate compared with free-living ones, as found in *Coccomyxa* (Zoller and Lutzoni 2003). Instead, a comparison of branch lengths of *Symbiochloris* and *Dictyochloropsis* revealed the opposite, i.e., that lichenized algae have longer branches than *Dictyochloropsis*.

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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:

Figure S1. Predicted secondary structures of the ITS2 transcripts of the 12 independent species-level lineages in the genus *Symbiochloris*.

Figure S2. Illustrated key for species determination.

Table S1. Species used in the phylogenetic analysis of Figure 1, their strain numbers (where available) and GenBank accession numbers.

Appendix S1. Alignment file of 92 18S rRNA gene sequences of aeroterrestrial green algae previously assigned to *Dictyochloropsis* and other members of the green algal class Trebouxiophyceae, in fasta format.

Appendix S2. Concatenated 18S-*rbcL* alignment of 38 *Dictyochloropsis* s.str. and *Symbiochloris* strains, in phylip format.

Appendix S3. Alignment file of 6 18S rRNA gene sequences of *Dictyochloropsis* s.str. (*Dictyochloropsis* clade I sensu Dal Grande et al. 2014) strains, in fasta format.

Appendix S4. Alignment file of 5 *rbcL* gene sequences of *Dictyochloropsis* s.str. (*Dictyochloropsis* clade I sensu Dal Grande et al. 2014) strains, in fasta format.

Appendix S5. Alignment file of 32 18S rRNA gene sequences of *Symbiochloris* (*Dictyochloropsis* clade II sensu Dal Grande et al. 2014) strains, in fasta format.

Appendix S6. Alignment file of 32 *rbcL* gene sequences of *Symbiochloris* (*Dictyochloropsis* clade II sensu Dal Grande et al. 2014) strains, in fasta format.