Chloroplast morphology and pyrenoid ultrastructural analyses reappraise the diversity of the lichen phycobiont genus *Trebouxia* (Chlorophyta)

Cesar D. Bordenave [a,b], Lucia Muggia [b,*], Salvador Chiva [a], Steve D. Leavitt [c], Pedro Carrasco [d], Eva Barreno [a]

[a] *Instituto "Cavanilles" de Biodiversidad y Biología Evolutiva (ICBiBE), Botánica, Fac. CC. Biológicas, Universitat de València, 46100 Burjassot, Valencia, Spain*
[b] *University of Trieste, Department of Life Sciences, via L. Giorgieri 10, 34127 Trieste, Italy*
[c] *Department of Biology and M. L. Bean Life Science Museum, Brigham Young University, Provo, UT, USA*
[d] *Instituto de Biotecnología y Biomedicina (BIOTECHMED), Universitat de València, 46100 Burjassot, Valencia, Spain*

ARTICLE INFO

Keywords:
Axenic culture
Microalgae
Microscopy
Species-level lineage
Symbiosis

ABSTRACT

Trebouxiophyceae is a wide class of green algae comprising coccoid and elliptic unicells, filaments, blades and colony-forming species that occur in diverse terrestrial and aquatic environments. Within this class, the genus *Trebouxia* Puymaly is among the most widespread lichen phycobionts worldwide. However, the 29 formally described species based on the combination of morphological traits and genetic diversity woefully underrepresented the overall species-level diversity recognized in the genus. In *Trebouxia*, reliable differentiation and characterization of the species-level lineages can be achieved by studying the diversity of key diagnostic features of pyrenoid ultrastructure and chloroplast morphology of axenically grown algal cultures. Here, we used transmission electron microscopy (TEM) coupled with confocal laser scanning microscopy (CLSM) to analyze the pyrenoid and the chloroplast of 20 *Trebouxia* species-level lineages grown directly on solid agar medium and on cellulose-acetate discs laid over the agar medium. With the new, detailed morphoanatomical characterization of these species-level lineages, we reappraise *Trebouxia* taxonomy in light of the most recent phylogenetic delimitation provided by Muggia et al. (2020).

1. Introduction

Trebouxiophyceae is a wide class of algae comprising coccoid and elliptic unicells, filaments, blades and colony-forming species that occur in diverse terrestrial and aquatic environments. Within the class, representatives have evolved different lifestyles and physiological performances, and some of them represent valuable research targets because of their biotechnological potential [1]. Some species have lost their photosynthetic capacity, evolving parasitic heterotrophic lifestyles [2–4], while others developed the capacity of living together with fungi. Among these algal symbionts, some have become the frequent photosynthetic partners (i.e., the photobionts) in lichen symbioses [5]. These latter are represented by the two large genera – *Trebouxia* Puymaly and *Asterochloris* Tschermak-Woess (Trebouxiales, Trebouxiaceae), which are the most species-rich lichen-forming, terrestrial, microalgal lineages [6–11].

Although lichens are among the best examples of symbiotic associations, so far research has mainly been directed towards the fungal partner, i.e., the mycobiont, the symbiont most responsible for the phenotype characterizing lichen diversity [12]. The mycobiont builds a three-dimensional structure of hyphae in which photobiont cells are enwrapped extracellularly, forming the lichen thallus, a true microecosystem where also other microorganisms are hosted [12,13]. In the past ten years, however, lichenologists and phycologists have increasingly focused their interest to the lichen photobionts and numerous studies shed light on an unexpected genetic diversity. In addition, the intrathalline coexistence of various microalgal lineages is a frequent event in lichen thalli ([14,58], and it has been suggested that the range of associated phycobionts could be influenced by thallus morphology [15].

The study of the two common phycobiont genera *Trebouxia* and *Asterochloris* has provided rewarding insight into understanding lichen symbioses and their diversity, both from ecological and evolutionary perspective. In particular, the lichen-forming, green algal genus *Trebouxia* is among the most widespread phycobionts, and members of this genus associate with a broad range of lichen-forming fungi, estimated to

* Corresponding author at: University of Trieste, Department of Life Sciences, via L. Giorgieri 10, 34127 Trieste, Italy.
E-mail address: lmuggia@units.it (L. Muggia).

Accepted 8 November 2021

be ca. 80% of lichen-forming fungi in temperate regions and more than 20% of all lichen-forming fungi worldwide [6,16]. To date, 29 *Trebouxia* species have been described based on the combination of morphological traits and genetic diversity ([9], and references therein). The work of Leavitt et al. [17] paved the way for a phylogenetic re-ordination of the several species-level lineages of the lichenized *Trebouxia*, further illustrating that diversity in this genus is far from being fully characterized. More recently the diversity and the evolutionary relationships of these species-level lineages were reappraised by Muggia et al. [6] who assembled DNA sequence data from over 1600 specimens, compiled from a range of sequences from previously published studies, axenic algal cultures, and lichens collected from poorly sampled regions. From this taxon sampling the authors selected representatives of the currently known genetic diversity and inferred a phylogeny from multi-locus sequence data (ITS, rbcL, cox2). The multi-locus phylogenetic reconstructions confirmed four major clades within *Trebouxia*; these are named after the representative species characterized by certain major morphoanatomical differences (as reported in the first morphological classification by [7]) and corresponded to the clades previously recovered using ITS data alone ([18–20]; and revised by [9]). Currently these clades are: clade A arboricola/gigantea-type, clade C corticola-type, clade I impressa/gelatinosa-type and clade S simplex/jamesii-type. Recently, some *Trebouxia* algae in clade S, specifically those associating with the lichen-forming fungus *Cetrariella delisei,* were provisionally segregated into the new clade 'D', supported by phylogenetic analyses based on four loci but still lacking a morphological characterization [21].

Notwithstanding their comprehensive analysis, Muggia et al. [6] highlighted that within each major lineage the formally described species woefully underrepresent the overall species-level diversity in the genus *Trebouxia,* providing an important impetus and a phylogenetic reference dataset for more reliably characterizing the diversity in this group of lichenized algae. The authors also stressed that an integrative taxonomic approach, combining morphological and physiological data from axenic cultures with genetic data, will be crucial to establish a robust, comprehensive taxonomy for *Trebouxia*. To this end, developing axenic cultures of representative *Trebouxia* phycobionts diversity is essential to reliably analyze morphological and physiological traits of putative species-level lineages. This first step is important to correlate the in vitro traits with those exhibited in the symbiotic state inside the lichen thallus [22,23].

Due to the relatively low rate of successful isolations of lichen photobionts, a clear characterization of the majority species-level lineages based on morphological and ultrastructural traits has been neglected. Therefore, to-date only the work of Friedl et al. [7,8] and a few previous and past studies by Ettl & Gärtner [24,25] and Gärtner & Ingolic [26,27] on specific lichen species, are available as reference for morphological and ultrastructural characterization of *Trebouxia* species. However, these works have become obsolete since the formal splitting of the genus *Trebouxia* into the two sister genera *Trebouxia* and *Asterochloris* [10] and the recent description of some new *Trebouxia* species [28]. Friedl's publication [8], though, proposed a set of putatively reliable traits for species recognition centered on pyrenoid ultrastructure and chloroplast morphology that potentially can be studied in all *Trebouxia*.

Indeed, *Trebouxia* cells contain a massive, axial, diversely lobed chloroplast which occupies almost the whole volume of the cytoplasm and usually contains a single pyrenoid [7,8,29]. The pyrenoid is a peculiar electron-dense protein matrix that has evolved in the chloroplast of unicellular photosynthetic microalgae. The protein matrix of the pyrenoid consists of the enzyme D-ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) and, in nearly all algae, is traversed by membranous invaginations of the stromatic thylakoids, through which CO_2 is delivered [8]. This structure has evolved in the Kingdom Plants to cope with the decreasing concentration of CO_2 caused by the colonization of the earth surface by land plants [30]. The carbon concentration mechanism that takes place in the pyrenoid improves the CO_2 fixation by the Rubisco, limiting the CO_2 leakage and preventing Rubisco from reacting with O_2 [31]. In the past few years pyrenoid research has gained interest, especially in the field of pyrenoid development in algae and pyrenoid establishment in vascular plants [32,33]. Recent genomic studies of the microalgae *Trebouxia* sp. TR9 identified proteins involved in carbon uptake, suggesting that this microalga may own utilize carbon concentration mechanisms similar to C3 and C4/CAM plants [34].

In particular, the structure and the extension of the chloroplast lobes, and the distinct arrangement of thylakoid and osmiophilic pyrenoglobules in the pyrenoid of *Trebouxia* have been regarded as key diagnostic characters [7–9,20,22,24,25]. Subsequent studies strengthen the congruence between ultrastructural variation and molecular data (as revised by [9]), while others even emphasized the perspective that the substantial morphological variation in pyrenoid structure observed within *Trebouxia* species is likely the result of genetically controlled polymorphisms or environmentally induced plasticity [35].

Here, we used transmission electron microscopy (TEM) coupled with confocal laser scanning microscopy (CLSM) to study the pyrenoid ultrastructure and the chloroplast morphology of 20 *Trebouxia* cultured strains axenically grown directly on solid agar medium and on cellulose-acetate discs laid over the agar medium. Each strain corresponds to a distinct, putative species-level lineage. For each, we provide a detailed species-specific characterization of pyrenoid ultrastructure and chloroplast morphology and present it as reference for a reliable species-level lineage recognition in the genus *Trebouxia*. With the new morphoanatomical characterization, we reappraise and implement the classification of *Trebouxia* species in accordance with the most updated phylogenetic delimitation provided by Muggia et al. [6]. In performing our analyses and in naming both pyrenoid and chloroplast types, we recognize the original classifications of Friedl et al. [7,8] for pyrenoid-types and that of Škaloud et al. [10] for the chloroplast-types, in addition to introducing new entries.

2. Materials and methods

2.1. Algae strains and growth conditions

We analyzed 20 *Trebouxia* strains, each corresponding to a distinct species-level lineage. Information about strain identity, species authorities, and their origin is reported in Table 1. Strains are deposited and maintained as living cultures at the Symbiotic Algal collection from the University of Valencia (ASUV, https://www.asuvalgae.com). A single strain of each *Trebouxia* species was selected for the microscopy analyses. Information about strain identity, species authorities, and their origin is reported in Table 1. Microalgae strains were maintained in Petri dishes on axenic solid Bold's Basal Medium (BBM) [36,37] for 21 days, strictly following the standardized growth conditions proposed for *Trebouxia* [38]. A subsample of each culture was taken for molecular analyses and genetic identification. Fifty μl of each suspended microalgae culture were applied directly over solid BBM or over acetate discs deposited on the same medium (according to [39]). Growth chamber conditions for the cultures were 20 °C under a 12:12 h light:dark cycle (25 μmol photons m^{-2} s^{-1}).

2.2. DNA extraction, amplification and sequencing

DNA was extracted from axenic algae cultures using a DNAeasy Plant Mini Kit following manufacturer's instructions (http://www.qiagen.com). The primers ITS1T and ITS4T [40] were used as forward and reverse primer, respectively, to amplify the algal ITS region (ITS1, 5.8S, ITS2). PCR cycling parameters followed a 66–56 °C touchdown cycle described in Lindblom & Ekman [41]. Complementary strands were sequenced from cleaned PCR products with the same primers used for amplification by Sanger sequencing (http://www.stabvida.com/).

A multiple sequence alignment (MSA) was prepared using the program MAFFT v7.475 [42] with the newly obtained *Trebouxia* ITS

Table 1
List of *Trebouxia* species analyzed in this study: phylogenetic affiliation (clade A-S) according to Muggia et al. [6], species name and authorities, strain ID, original culture collection ID and original lichen sample are reported. The nomenclature of the taxa follows the AlgalBase database (https://www.algaebase.org/). Culture collections acronyms are the following: ASUV - Symbiotic Algal collection from the University of Valencia (https://www.asuvalgae.com/); SAG - Culture collection of algae at the University of Göttingen (http://www.epsag.uni-goettingen.de/html/sag.html); UTEX - Culture collection at the University of Austin, Texas (http://www.bio.utexas.edu/research/utex/); CCAP - Culture collection of algae and protozoa, UK (https://www.ccap.ac.uk/).

Clade affiliation	Trebouxia species name	Strain ID (GeneBank acc. no.)	Original culture ID	Original lichen sampl0e
Clade A	*T. decolorans* Ahmadjian	ASUV 41 (OK577843)	UTEX B781	*Buellia punctata* from West Boyalston, Massachusetts, USA
Clade A	*T. crenulata* Archibald	ASUV 83 (OK577844)	CCAP 219/2	*Xanthoria aureola* from Delft, Netherlands
Clade A	*T. aggregata* (Archibald) Gärtner	ASUV 73 (OK577845)	SAG 219-1d	*Xanthoria parietina* from Delft, Netherlands
Clade A	*T. arboricola* Puymaly	ASUV 57 (OK577846)	SAG 219-1a	Delft, Netherlands
Clade A	*T. cretacea* Voytsekhovich & Beck	ASUV 133 (OK577847)		*Buellia zoharyi* from Titulcia, Madrid, Spain
Clade A	*T. showmanii* (Hildreth & Ahmadjian) Gärtner	ASUV 61 (OK577848)	SAG 2009	*Lecanora hageni* from Ohio, USA
Clade A	*T. asymmetrica* Friedl & Gärtner	ASUV 128 (OK577849)	SAG 48.88	*Diploschistes albescens* from, Sa. de Roses, Catalunya, Spain
Clade A	*T. gigantea* (Hildreth & Ahmadjian) Gärtner	ASUV 51 (OK577850)	UTEX 2231	*Caloplaca cerina* from Ohio, USA
Clade A	*T. incrustata* Ahmadjian ex Gärtner	ASUV 87 (OK577851)	UTEX 784, CCAP 219/6	*Lecanora dispersa* from Cambridge, Massachusetts, USA
Clade A	*T. jamesii* (Hildreth & Ahmadjian) Gärtner	ASUV 129 (OK577852)	SAG 2103	*Lecanora hageni* from Devil's Bridge, Wales, UK.
Clade A	*Trebouxia* sp. TR9	ASUV 44 (OK577853)		*Ramalina farinacea* from El Toro, Castellón, Spain
Clade S	*T. simplex*	ASUV 43 (OK577854)		*Umbilicaria* sp. from Tenerife, Spain
Clade S	*T. australis* Beck	ASUV 59 (OK577855)	SAG 2205	*Brindabella* Range, Australia.
Clade S	Trebouxia sp. S08	ASUV 143 (OK577856)		*Parmelia sulcata* from Soria, Spain
Clade I	*T. impressa* Ahmadjian	ASUV 52 (OK577857)	UTEX 893	*Physcia stellaris* from USA
Clade I	*T. flava* P.A. Archibald	ASUV 84 (OK577858)	UTEX 181, CCAP 219/1C	*Physcia pulverulenta* from Delft, Netherlands
Clade I	*T. potteri* Ahmadjian ex Gärtner	ASUV 86 (OK577859)	UTEX 900, CCAP 219/7	*Lecanora rubina* from Berlin, Massachusetts, USA
Clade I	*T. anticipata* Ahmadjian ex Archibald	ASUV 49 (OK577860)	UTEX 904	*Parmelia rudecta* from Franconia Notch, New Hampshire, USA
Clade C		ASUV 80 (OK577861)	UTEX 909	Isolated from bark from USA
	T. corticola (P.A. Archibald) Gärtner			
Clade C	*T. crespoana* Barreno, Molins, Moya & Škaloud	ASUV 132 (OK577862)		*Parmotrema pseudotinctorum* from Breña Baja, Canary Islands, Spain

sequences and the sequence data assembled as described in Muggia et al. [6], which included sequences of reference strains . We implemented the FFTNS-i alignment algorithm and '200PAM / K = 2' scoring matrix, with an offset value of 0.0, using the 'Leave gappy regions' setting, and the remaining parameters set at default values. Based on the results of the initial alignment and exploratory phylogenetic reconstructions, sequences were divided into four groups corresponding to the four major *Trebouxia* clades, i.e. clade 'A', clade 'C', clade 'I' and clade 'S' as recognized in Muggia et al. [6]. Clade-specific MSAs of ITS sequences were performed in MAFFT using the G-INS-I alignment algorithm (Fig. S1a–d).

The phylogenetic analyses were performed for each clade using the maximum likelihood approach (ML, [43,44]) implemented in RAxML v.8.2.10 [45] applying the GTRGAMMA model and running 1000 bootstrap replicates. The phylogenetic trees were visualized in TreeView v1.6.6 [46].

2.3. Transmission electron microscopy

Experiments were carried out growing the microalgae strains directly on solid BBM or over acetate discs. For each growth condition, a single colony was prepared for the TEM analyses following fixation and dehydration protocols as described in Molins et al. [22,47]. In brief, samples were fixed in 2% Karnovsky fixative for 12 h at 4 °C, washed three times for 15 min with 0.01 M PBS (pH 7.4), and postfixed with 2% OsO_4 in 0.01 M PBS (pH 7.4) for 2 h at room temperature. After washing in 0.01 M PBS, pH 7.4, the samples were dehydrated at room temperature in a graded series of ethanol starting at 50% and increasing to 70%, 95% and 100% for no less than 20–30 min at each step. The fixed and dehydrated samples were embedded in Spurr's resin according to the manufacturer's instructions (http://www.emsdiasum.com/microscopy/technical/datasheet/14300.aspx). Ultra-thin sections, 80 nm thick, were cut with a diamond knife (Diatome ultra 458), and mounted on 100 mesh copper grids as described in Moya et al. [48], and then stained with 10% uranyl acetate and 0.1% lead citrate using the 'Synaptek Grid-Stick Kit'. Sections were observed at 80 kV under a JEOL JEM-1010 microscope (Jeol, Peabody, MA, USA). Images were obtained using an Olympus MegaView III camera and processed with Fiji distribution of ImageJ [49]. For each sample at least 20 mature vegetative cells were observed and representative images of the whole cell and a detail of the pyrenoid ultrastructure were acquired.

2.4. Confocal laser scanning microscopy

Culture experiments were carried out using three different growth conditions: in tubes containing liquid BBM, directly on solid BBM or over acetate discs deposited on the same medium. A single colony of each growth condition was prepared for the CLSM analysis. The whole algal colony was scraped off from the solid medium, or the acetate discs, with a sterilized loop and re-suspended in 30 µl of liquid 3 N BBM. 15 µl of each population were placed on plates with a thin layer of 1% agar in sterile water, air dried and placed upside down over a 35 mm imaging dish suitable for inverted microscopy. For the liquid BBM experiments,

15 µl of each liquid culture were directly used as described above. An Olympus FLUOVIEW FV1000 laser scanning confocal microscope was used with a 405 nm excitation laser. Fluorescence emitted from 650 to 750 nm was collected to observe chlorophyll autofluorescence, thus recovering the chloroplast layers. For each sample at least 20 mature vegetative cells were observed, and representative images were acquired. A series of images were captured with a separation of 0.4 µm. The image stack was preprocessed to remove noise and then analyzed using z-projection tool and volume viewer with Fiji distribution of ImageJ [49].

3. Results

The phylogenetic analyses performed for each *Trebouxia* clade fully corresponded to the reference phylogeny provided by Muggia et al. [6]. For cultures recovered in each of the four clade-specific phylogenies (clades A, C, I and S), we report the main pyrenoid and chloroplast types (Figs. 1–4, Table 2) and their intraspecific variability at each species-level lineages (Supplementary Material, Figs. S1a–d, S2–S7).

3.1. Diversity of pyrenoid ultrastructure

Based on different arrangements and forms of thylakoid lamellae within the pyrenoid matrix, a total of six main different types of pyrenoids were observed among the 20 *Trebouxia* cultures analyzed (Table 2). They are either named following the original nomenclature used by Friedl et al. [8], or after one representative species recognized for any new pyrenoid-type. Each pyrenoid-type is characteristic of either a single species so far, or of a group of species; for each species we recovered only one pyrenoid-type (Table 2). There were no substantial differences in the pyrenoid ultrastructure between algal cells grown on agar medium or acetate discs (Figs. 1–4; Supplementary Figs. S2–S7).

Decolorans-type pyrenoids are characterized by long parallel tubules traversing the pyrenoid matrix which is thicker than the tubules (Table 2; Fig. 1a,b). We observed pyrenoglobules always present in moderate to high numbers inside the pyrenoid matrix next to the tubules. In general, the central mass of this pyrenoid is rather thin. This pyrenoid type was observed exclusively in *T. decolorans* of clade A (Supplementary Material Fig. S1), which furthermore presented 2–4 pyrenoids per chloroplast (Supplementary Material Fig. S2a–d).

Crenulata-type pyrenoids are characterized by long branched tubules meandering through the pyrenoid matrix with a matrix thicker than the tubules (Fig. 1e,f; Table 2). We observed few pyrenoglobules present inside the pyrenoid matrix next to the tubules. This pyrenoid type was observed exclusively in *T. crenulata* belonging to clade A (Supplementary Material Fig. S1), consistently with a single pyrenoid per chloroplast (Supplementary Material Fig. S2g–j).

Gigantea-type pyrenoids are characterized by short-branched tubules perforating the pyrenoid matrix, with tubules as thick as the matrix (Figs. 1i,j,m,n,q,r; 2a,b,e,f,i,j, m,n; Table 2). We observed pyrenoglobules always present in variable numbers inside the pyrenoid matrix or at the margins of the pyrenoid. This pyrenoid type was observed only in species of clade A (Supplementary Material Fig. S1), i.e., *T. aggregata* (Fig. 1i–j), *T. arboricola* (Fig. 1m,n), *T. cretacea* (Fig. 1q,r), *T. showmanii* (Fig. 2a,b), *T. gigantea* (Fig. 2e,f), *T. asymmetrica* (Fig. 2i,j) and *T. incrustata* (Fig. 2m, n). The major difference between these species consists in the number of pyrenoids and pyrenoglobules. *Trebouxia aggregata, T. arboricola, T. showmanii, T. gigantea* and *T. incrustata* presented a single pyrenoid (Supplementary Material Figs. S2m–p, s–t, S3a–b, k–n, S4c–f and i–l), whereas we counted 4–17 and 2–6 pyrenoids per chloroplast in *T. cretacea* and *T. asymmetrica* (Supplementary Material Fig. S3e–h, q–t), respectively. In *T. showmanii* and *T. asymmetrica* pyrenoglobules were abundant and arranged in a row next to the invaginations of the tubules. In *T. incrustata*, the pyrenoid showed a thinner matrix than that in the regular gigantea-type, resulting in tubules thicker than the matrix.

Impressa-type pyrenoids are characterized by radial straight unbranched tubules penetrating the pyrenoid matrix, appearing either long or short depending on the orientation of the section (Figs. 2q,r; 3a,b,e,f,i, j,m,n,q,r; 4a,b,e,f). The pyrenoid matrix is always thicker than the tubules. Pyrenoglobules are always present, in moderate to high numbers inside the matrix (Table 2). This pyrenoid-type seems to be the most widespread among the *Trebouxia* phylogeny, being present in three of the four major clades (Supplementary Material Fig. S1a, c, d). It was observed in two closely related members of clade A (*T. jamesii* and *Trebouxia* sp. TR9; Figs. 2q,r; 3a,b; Supplementary Material Fig. S1), in all members of clade S here analyzed (*T. simplex*, *T. australis* and *Trebouxia* sp. S08; Fig. 3e, f, i, j, m, n; Supplementary Material Fig. S1d) and in clade I for *T. impressa, T. flava* and *T. potteri* (Figs. 3q, r; 4a, b, e, f; Supplementary Material Fig. S1c). *T. impressa* had only a single pyrenoid in their chloroplast, while in general the analyzed strains of the other species bore multiple pyrenoids (Table 2; Supplementary Material Figs. S4o–r; S5a–d, g–j, m-p, s–t; Fig. 6a,b, e–h, k–n, q–t). *T. jamesii*, *Trebouxia* sp. TR9, *Trebouxia* sp. S08 and *T. impressa* presented pyrenoglobules in moderate numbers arranged in a row next to the invaginations of the tubules, whereas *T. simplex*, *T. australis*, *T. flava* and *T. potteri* showed a high number of pyrenoglobules less clearly arranged and spread throughout the matrix. An electron lucent region was observed in the central part of the pyrenoid matrix when the algae were grown on acetate discs. This region was not observed in the pyrenoids of *T. simplex* and *Trebouxia* sp. S08; instead, it was present in the pyrenoid of *T. impressa* also when grown directly on the medium.

Anticipata-type pyrenoids form a single mass occupying most of the central body of the chloroplast (Fig. 4i,j; Table 2; Supplementary Material Fig. S7c–f). Although this pyrenoid resembles a gigantea-type, it is surrounded by unstacked thylakoid lamellae arranged almost perpendicular to each other (Fig. 4i–j; Table 2). Pyrenoglobules were highly variable in number with no clear distribution but always inside the pyrenoid matrix. This type was exclusively found in *T. anticipata* (Fig. 4i–j) belonging to clade I (Supplementary Material Fig. S1c).

Corticola-type pyrenoids sometimes are inconspicuous and hardly detectable. When clearly visible, the pyrenoid is characterized by a matrix traversed by membranous inclusions bearing a close structural resemblance to the thylakoid lamellae (Table 2; Fig. 4m–n, q–r). Two distinct regions of irregular shapes were observed, one electron dense that resembled the regular pyrenoid matrix and one electron lucent that resembled the one observed in some impressa-type pyrenoids. Pyrenoglobules were not associated with the matrix, instead distributed in the chloroplast stroma, surrounding the pyrenoid. Starch grains were usually adjacent to the pyrenoid matrix, forming a starch sheath which consists of a few large, curved plates. This pyrenoid-type was exclusive of clade C (Supplementary Material Fig. S1b), as it was found in *T. corticola* (Fig. 4m–n) and *T. crespoana* (Fig. 4q–r), here analyzed. Both species presented a single pyrenoid (Supplementary Material Fig. S7i–l, o–r).

3.2. Diversity of chloroplast morphology

Mature vegetative cells of each *Trebouxia* species showed only one chloroplast-type under the growing conditions applied here. *Trebouxia* chloroplasts were characterized by a central plastid body from which lobes of rather short length emerge. Differences in lobe arrangement and lobe termination types allowed the identification of five main different chloroplast types, including their intraspecific variability, among the 20 *Trebouxia* species analyzed (Table 2). They are either named following the original nomenclature used by Škaloud et al. [10] for *Asterochloris*, or new names were introduced based on the peculiar novel morphology identified.

Deeply lobed-type chloroplasts [10] have long, branched, or unbranched lobes emerging directly from a thin chloroplast layer (Fig. 1c, d; Table 2); elongated lobes are sometimes crossed by perforations. This chloroplast type was observed exclusively in *T. decolorans* (Fig. 1c,d;

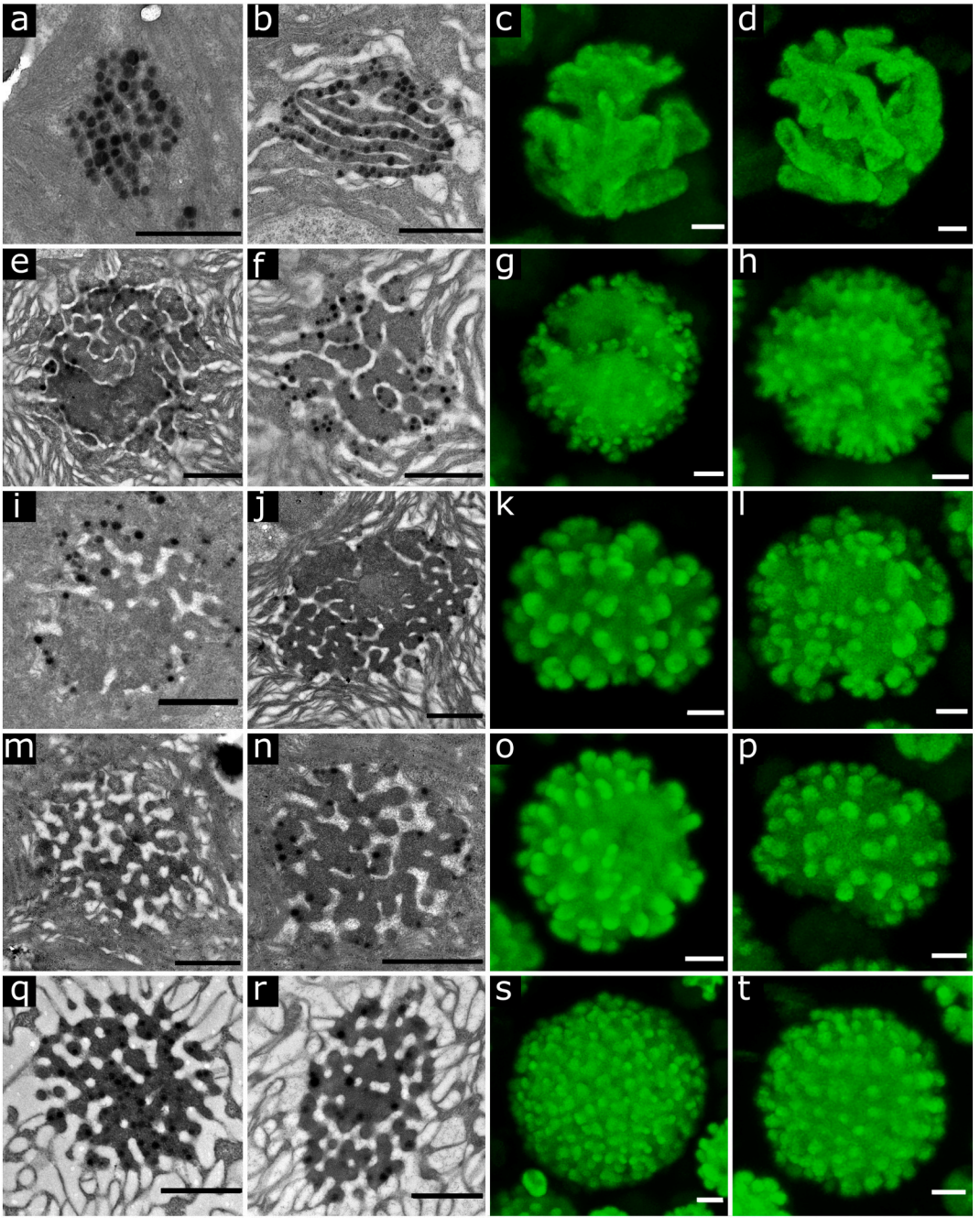


Fig. 1. Pyrenoid ultrastructure and chloroplast morphology in *Trebouxia* species. Each row consists of images corresponding to a single species. First and second columns correspond to TEM micrographs of pyrenoid ultrastructure. Third and fourth columns correspond to CLSM chloroplast reconstructions. First and third columns are algae grown on agarized BBM, second and fourth columns are algae grown on acetate discs over the agarized BBM. a–d, *T. decolorans;* e–h, *T. crenulata;* i–l, *T. aggregata;* m–p, *T. arboricola;* q–t, *T. cretacea*. Scale bar (a–t) = 1 μm.

Supplementary Material Fig. Sa–d) of clade A (Supplementary Material Fig. S1).

Crenulate-type chloroplasts [10] have a central, massive chloroplast with a regularly lobulated surface (Table 2). This chloroplast type was exclusive of clade A and is found in the species *T. crenulata* (Fig. 1g, h), *T. aggregata* (Fig. 1k, l), *T. arboricola* (Fig. 1o, p), *T. cretacea* (Fig. 1s, t) and *T. showmanii* (Fig. 2a, b; Supplementary Material Fig. S1). The chloroplasts of these species differ mainly in the type of the lobe terminations. *T. crenulata* (Fig. 1g,h; Supplementary Material Fig. S2g–j) and *T. showmanii* (Fig. 2c,d; Supplementary Material Fig. S3k–n) possess branched elongated 'tree-like' lobes, particularly abundant in *T. crenulata* giving the chloroplast a 'forest-like' aspect. The chloroplast of *T. aggregata* (Fig. 1k,l; Supplementary Material Fig. S2m–p) and *T. arboricola* (Fig. 1o,p; Supplementary Material Figs. S2s,t; and S3a,b) presented abundant lobes slightly branched, that seemed like two lobes would overlap. In *T. cretacea* (Fig. 1s,t; Supplementary Material Fig. S2e–h), the chloroplast was well distinguishable from the other strains as it had simple unbranched lobes which were the smallest in relation to the chloroplast size. Some of the strains bearing a crenulate-type chloroplast showed a particular chloroplast morphology when grown on liquid BBM. Under this condition, chloroplasts of *T. crenulata, T. aggregata* and *T. arboricola* developed elongated lobes, very thin in the case of *T. crenulata* and *T. arboricola*, over which secondary small lobes developed giving the chloroplast a 'tassel trim-like' aspect

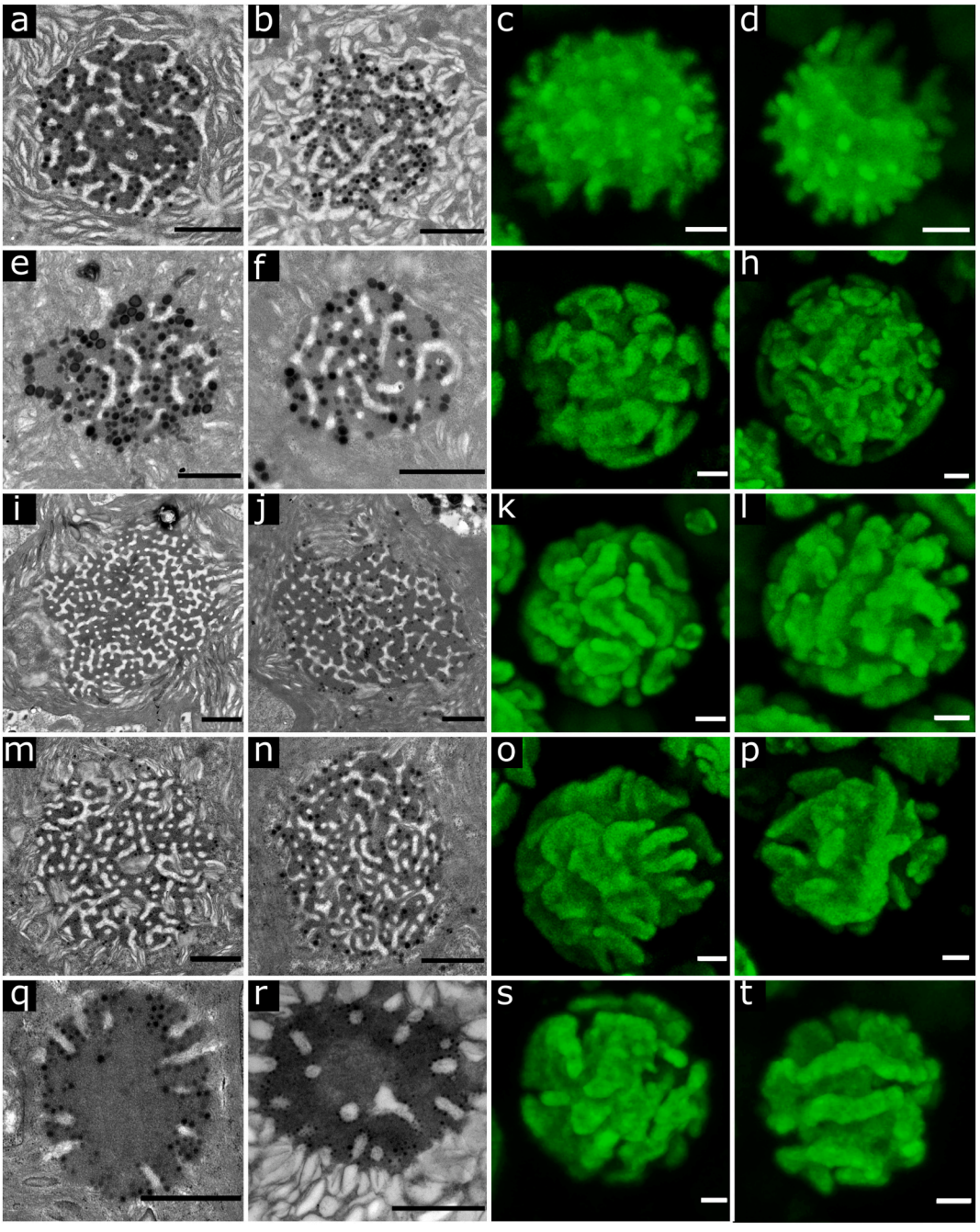


Fig. 2. Pyrenoid ultrastructure and chloroplast morphology in *Trebouxia* species. Each row consists of images corresponding to a single species. First and second columns correspond to TEM micrographs of pyrenoid ultrastructure. Third and fourth columns correspond to CLSM chloroplast reconstructions. First and third columns are algae grown on agarized BBM, second and fourth columns are algae grown on acetate discs over the agarized BBM. a–d, *T. showmanii;* e–h, *Typhlogastrura asymmetrica;* i–l, *T. gigantea;* m–p, *T. incrustata;* q–t, *Telesonix jamesii.* Scale bar (a–t) = 1 μm.

(Supplementary Material Figs. S2k, l, q, r; and S3c, d).

Shallowly lobed-type chloroplasts [10] have a central mass from which lobes arise. This was the most frequently recovered chloroplast morphology in our set of strains, as we observed it in 11 species within clades A, I and S (Figs. 2-4; Supplementary Material Fig. S1a, c, d), i.e. in *T. asymmetrica* (Fig. 2g, h), *T. gigantea* (Fig. 2k, l), *T. incrustata* (Fig. 2o, p), *T. jamesii* (Fig. 2s, t), *Trebouxia* sp. TR9 (Fig. 3c, d), *T. simplex* (Fig. 3g, h), *T. australis* (Fig. 3k, l), *Trebouxia* sp. S08 (Fig. 3o, p), *T. impressa* (Fig. 3s, t), *T. flava* (Fig. 4c, d) and *T. potteri* (Fig. 4g, h). Most of these strains have elongated lobes meandering around the chloroplast surface, resembling the shape of a nut or a brain (Table 2). The only exception is the chloroplast of *T. asymmetrica* which possesses flat lobe terminations (Fig. 2g, h; Supplementary Material Fig. S3q–t). When these *Trebouxia* strains were grown in liquid BBM they generally maintained consistent chloroplast morphologies (Supplementary Material Figs. S4–S7), while only *T. australis* developed a chloroplast that resembled a tassel trim-like morphology (Supplementary Material Fig. S5q, r).

Curly lobed-type chloroplasts (new, this study) have a central mass from which superficial lobes arise; these are peculiar because it seems as elongated lobes and simple lobes overlaping each other (Fig. 4k, l; Table 2). Although this morphology may resemble the tassel trim-like lobes described above (lobes are one over the other), these lobes are clearly differentiated. This chloroplast type was observed only in *T. anticipata* (Fig. 4k,l; Supplementary Material Fig. S7c–f) of clade I

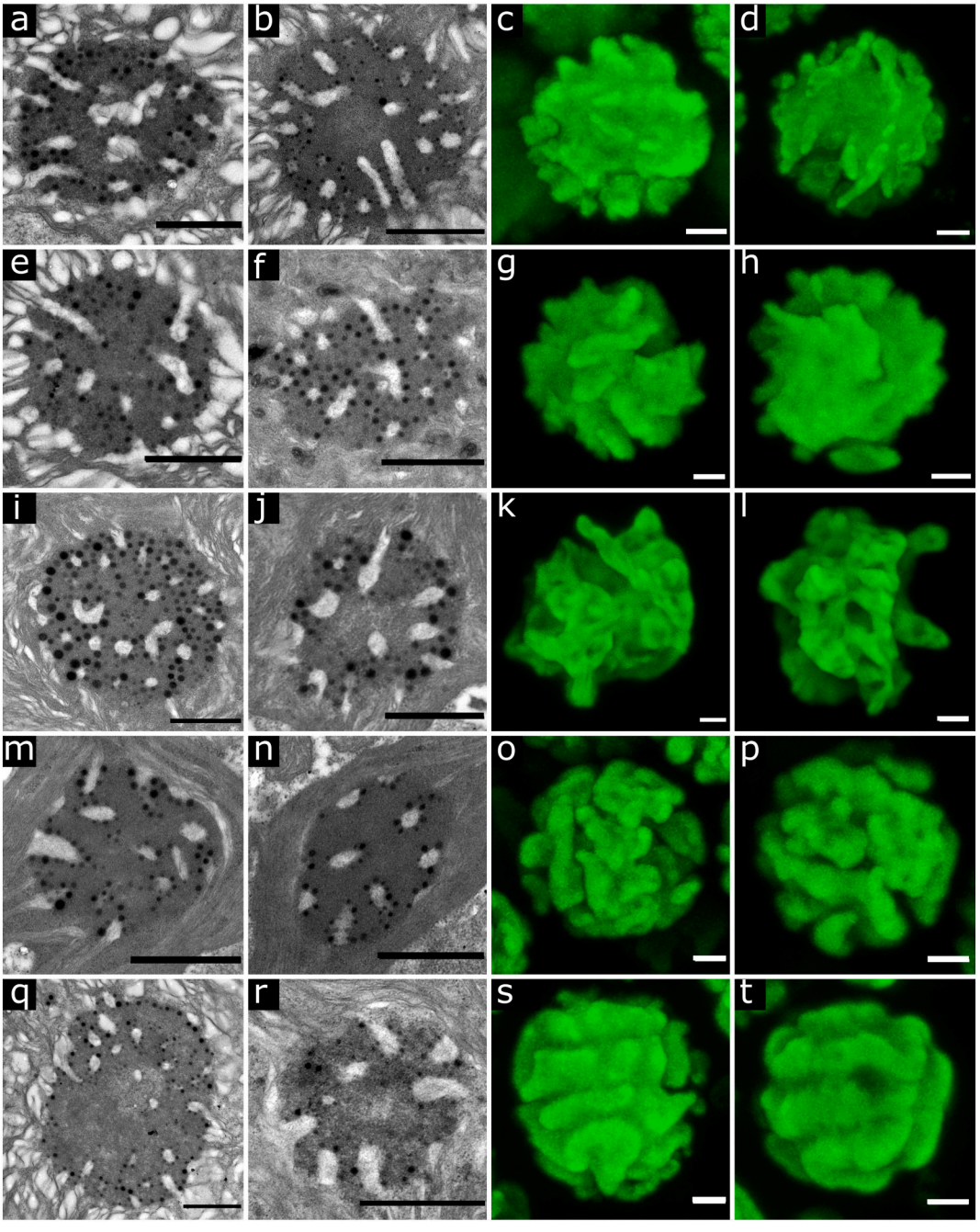


Fig. 3. Pyrenoid ultrastructure and chloroplast morphology in *Trebouxia* species. Each row consists of images corresponding to a single species. First and second columns correspond to TEM micrographs of pyrenoid ultrastructure. Third and fourth columns correspond to CLSM chloroplast reconstructions. First and third columns are algae grown on agarized BBM, second and fourth columns are algae grown on acetate discs over the agarized BBM. a–d, *Trebouxia* sp. TR9*;* e–h, *T. simplex*; i–l, *T. australis*; m–p, *Trebouxia* sp. S08*;* q–t, *T. impressa.* Scale bar (a–t) = 1 μm.

(Supplementary Material Fig. S1c).

Thin lobed-type chloroplasts (new, this study) have a central mass from which very thin elongated lobes arise and meander around the chloroplast surface (Fig. 4o, p, s, t; Table 2). This chloroplast type was observed exclusively in the two members of clade C analyzed (Supplementary Material Fig. S1b), i.e., *T. corticola* (Fig. 4o,p; Supplementary Material Fig. S7i–l) and *T. crespoana* (Fig. 4s,t, and Fig. S7o–r). These two strains were hardly distinguishable, although when grown on liquid BBM *T. crespoana* showed a crenulate-like chloroplast (Supplementary Material Fig. S7s–t) while *T. corticola* maintains the thin lobed morphology (Supplementary Material Fig. S7m–n).

4. Discussion

The morpho-anatomical traits of *Trebouxia* chloroplast were herewith revised to provide a detailed characterization of pyrenoid ultrastructure and chloroplast morphology to be used as reference for reliable species-level lineage recognition in this algal genus. *Trebouxia* cells contain a massive, axial, differently lobed chloroplast that occupies almost the entire volume of the cytoplasm and contain one or more pyrenoids (Figs. 1–4). These chloroplast characteristics clearly support the segregation of *Trebouxia* from the sister genera *Asterochloris* and *Vulcanochloris* Vancurová*,* whose chloroplast consists of a central layer from which long lobes extend [10,50], and from *Myrmecia* Printz, whose

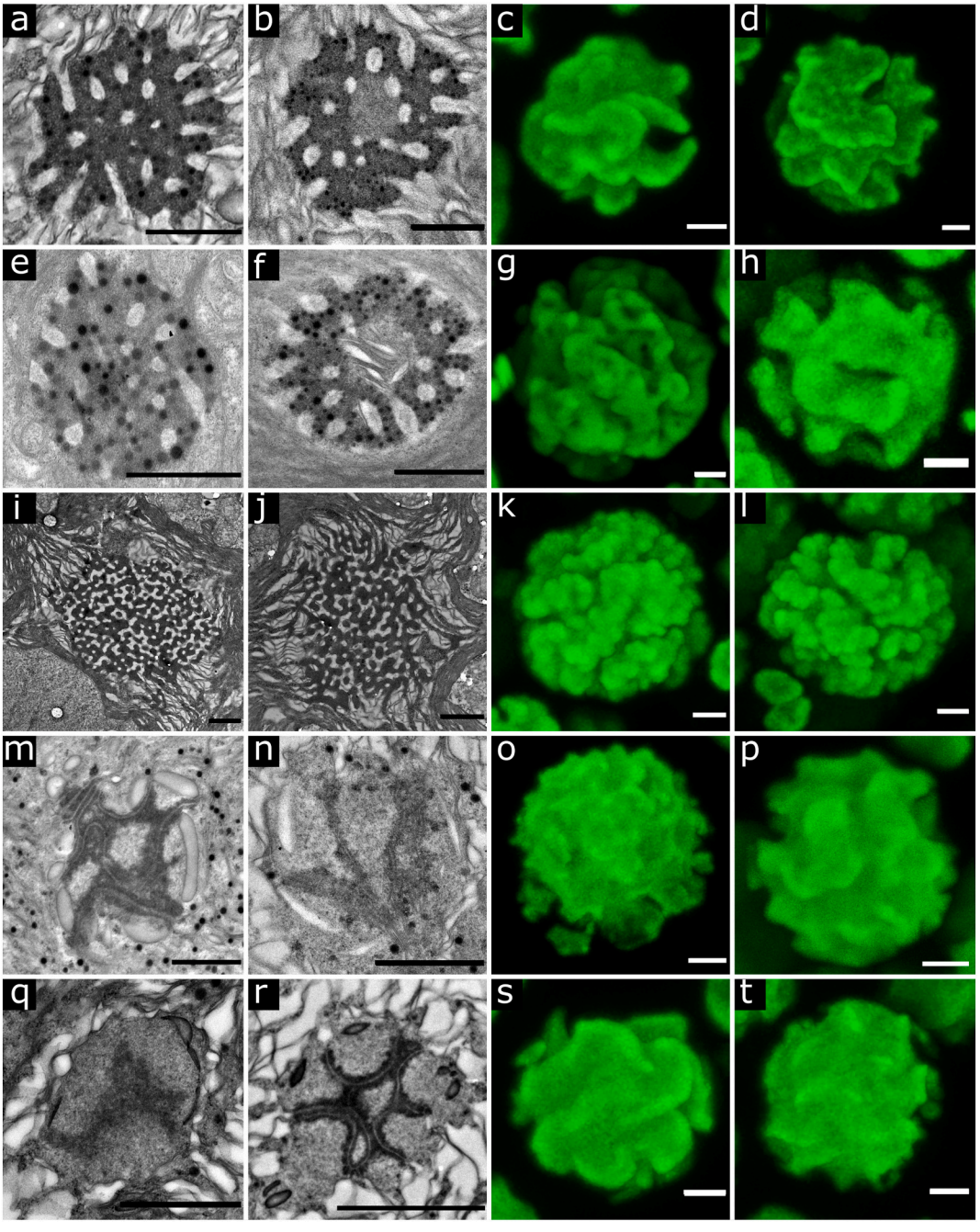
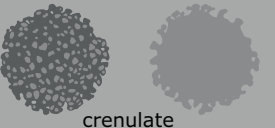
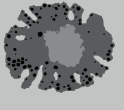
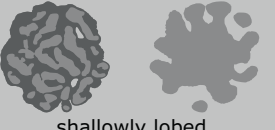
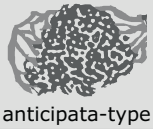
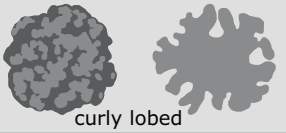
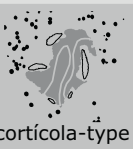


Fig. 4. Pyrenoid ultrastructure and chloroplast morphology in *Trebouxia* species. Each row consists of images corresponding to a single species. First and second columns correspond to TEM micrographs of pyrenoid ultrastructure. Third and fourth columns correspond to CLSM chloroplast reconstructions. First and third columns are algae grown on agarized BBM, second and fourth columns are algae grown on acetate discs over the agarized BBM. a–d, *T. flava;* e–h, *T.potteri;* i–l, *T. anticipata;* m–p, *T. corticola;* q–t, *T. crespoana.* Scale bar (a–t) = 1 μm.

cells present a bipartite cup–shaped parietal chloroplast without a pyrenoid [51]. We also provide further evidence that *Trebouxia* pyrenoid ultrastructure and chloroplast morphology are conserved when cells are grown either directly on solid BBM or on acetate discs laid over the solid BBM. Our results are in line with the original observation done by Friedl et al. [7,8], who reported conserved pyrenoid types even when *Trebouxia* species were grown on different culture media. Cellulose-acetate discs are ideal for short-term experiments because the mesh size of the disc enables the diffusion of the micronutrients through the disc itself and the algal growth can be easily monitored [52,53]. Furthermore, the approach using acetate discs facilitates changing the culture medium or assaying stressful conditions on mature colonies simply by removing the disc without disrupting the structure of the algal colonies [54–56]. Occasionally, the growth on acetate disc may be preferred to the direct growth on agar, as it was considered to better resemble the growth in the lichen thalli [39]. Notwithstanding, the cultivation of *Trebouxia* cells on solid BBM has been recommended as standard condition for subsequent morpho-anatomical analyses, estandarized to be performed 21 days after inoculation of the cells on the medium [38]. In the present analyses, we assert the validity of this protocol and highlight that the use of cellulose-acetate discs does not impair the development of pyrenoid ultrastructure and chloroplast morphology. Instead, the morpho-anatomical structures of *Trebouxia* chloroplasts can be distorted when cells are grown in liquid BBM. Although liquid culture media are

Table 2
T. asymmetrica.

Pyrenoid Schematic Drawing	*Trebouxia* species (Clade)	Chloroplast Schematic Drawing
 decolorans-type	***T. decolorans***[2-4] (A)	 deeply lobed
 crenulata-type	***T. crenulata***[1] (A)	 crenulate
 gigantea-type	*T. aggregata*[1] (A) *T. arboricola*[1] (A) *T. cretacea*[4-17] (A) *T. showmanii*[1] (A) *T. asymmetrica*[2-6] (A) ***T. gigantea***[1] (A) *T. incrustata*[1] (A)	
 impressa-type	*T. jamesii*[2-4] (A) *Trebouxia* sp. TR9[2-6] (A) *T. simplex*[1-3] (S) *T. australis*[1-5] (S) *Trebouxia* sp. S08[1-2] (S) ***T. impressa***[1] (I) *T. flava*[1-3] (I) *T. potteri*[1-4] (I)	 shallowly lobed
 anticipata-type	***T. anticipata***[1] (I)	 curly lobed
 cortícola-type	***T. corticola***[1] (C) *T. crespoana*[1] (C)	 thin lobed

commonly applied for obtaining substantial biomass in a shorter time, we advise against them, if morpho-anatomical analyses must be performed.

In general, *Trebouxia* species display a high diversity in terms of pyrenoid tubule arrangement, abundance and distribution of pyrenoglobules, and density and termination type of chloroplast lobes (Figs. 1–4). Analyzing these traits in the 20 selected species, it was possible to classify *Trebouxia* pyrenoids into six types and chloroplasts into five types (Table 2), and to define seven chloroplast architectures resulting from the combination of a pyrenoid and a chloroplast type. In doing this we identified three *Trebouxia* species characterized by their unique pyrenoid type, i.e., *T. decolorans*, *T. crenulata* and *T. anticipata*, while *T. decolorans* and *T. anticipata* are also unique for their chloroplast morphology. For these two species, the pyrenoid types were introduced as new and are distinguished by the peculiar arrangement of the tylakoid lamellae. In the decolorans-type these are parallel as tubules immersed in the pyrenoid matrix, while in anticipata-type they are unstacked lamellae surrounding the pyrenoid matrix. Both the decolorans- and anticipata-types clearly differentiate from the classification proposed by Friedl [7,8], in which the pyrenoid of *T. decolorans* was designate as arboricola-type, and that of *T. anticipata* was the gelatinosa-type.

Based on our results, we reappraised the arboricola-type sensu Friedl [7,8] and renamed it as crenulata-type after *T. crenulata*, as this species is the only one bearing this type of pyrenoid. In *T. arboricola* and *T. aggregata*, we recognize pyrenoids of the gigantea-type sensu Friedl [7,8]. The gigantea-type pyrenoid presents a wide spectrum of ultrastructure among the species owing it. Indeed, through a detailed inspection of the microphotographs it was possible to perceive a subtle variation in the distribution of the short-branched tubules perforating the pyrenoid matrix and the variable numbers of pyrenoglobules. However, this variation does not support any additional subdivision of types, rather, it represents the presence of high variability in the gigantea-type pyrenoid among *Trebouxia*.

We report here for the first time for the genus *Trebouxia* the presence of electron-lucent regions in the pyrenoid matrix. These electron-lucent regions, either single or multiple in the pyrenoids, are already known for the genus *Vulcanochloris*, frequently occurring as spherical to elongated regions [50]. In *Vulcanochloris*, these electron-lucent regions rarely are associated with several pyrenoglobules, while occasionally more than eight regions are formed within the pyrenoid matrix, and likely correspond to spherical pyrenoid incisions observed in a light microscope [50].

The morphological analysis of the chloroplasts allow us to distinguish five types that are here formally described for the first time for *Trebouxia*. Our classification is presented following the system introduced by Škaloud et al. [10] for the sister genus *Asterochloris* and finds only partial correspondence with the descriptions of chloroplast morphology originally proposed by Friedl [7,8] -who recognized nine morphologies for *Trebouxia* chloroplasts. We report here two new chloroplast types, i.e., the 'curly lobed' and the 'thin lobed'. These are clearly distinguishable variants of the rather commonly found shallowly lobed morphology, this latter characterized by elongated lobes meandering around the chloroplast surface, resembling the shape of a nut/brain. Alternatively, the crenulate lobed morphology resulted to be the one most diverse for that it concerns the type of lobe terminations. Indeed, similarly as for the pyrenoid gigantea-type, this chloroplast type presents subtle variations being the lobe more or less branched and elongated, conferring either a 'tree-like' or 'tassel trim-like' aspect.

We found unique correspondence/association of pyrenoid and chloroplast types only for *T. decolorans* and *T. anticipata*, while we identified a multiplicity of combinations (seven in total) between pyrenoid and chloroplast types which generates a great morpho-anatomical diversity. These combinations of traits can be better used to segregate species, than looking independently only at one cellular trait, i.e., either the chloroplast morphology or the pyrenoid ultrastructure. Although we are aware that the identification of new species-level lineages in *Trebouxia* should not be based merely on the diversity of chloroplast morphology and pyrenoid ultrastructure, we are confident that the analyses and the classification system presented here offers a reliable identification methodology. When pyrenoid and chloroplast types are considered in the light of the most updated (phylo)genetic delimitation provided by Muggia et al. [6] it is possible to assess the diversity of chloroplast architectures within each clade. Clade A encompasses the majority of previously recognized *Trebouxia* species and includes most axenically isolated taxa characterized for their physiological traits (as revised by [9]). The species-level lineages belonging to this clade analyzed here displayed the greatest diversity in terms of chloroplast and pyrenoid types, with five of the seven different combinations being represented within this clade (Table 2). Two subclades within clade A can be clearly distinguished, bearing either the same pyrenoid or chloroplast type: the first includes *Trebouxia* sp. TR9 and *T. jamesii* with a combination of multiple impressa-type pyrenoids per chloroplast and presenting a shallowly lobe chloroplast (Supplementary Material Fig. S1); the second includes *T. crenulata, T. arboricola* and *T. aggregata* with the same crenulate-type chloroplast but presents either a single crenulata-type or gigantea-type pyrenoid per chloroplast (Supplementary Material Fig. S1). The other species-level lineages in clade A are less closely related to the two above-mentioned subclades and share the same gigantea-type pyrenoid, which only in *T. asymmetrica* and *T. cretacea* is present as more than one per chloroplast. Two chloroplast types are identified in these species, i.e., the crenulate lobed and the shallowly lobed. Among these species, only *T. incrustata* displayed the chloroplast with flat lobe terminations (Fig. 2). Although *T. showmanii* and *T. cretacea* also shared the same chloroplast architecture, *T. cretacea* possess unique features among the strains analyzed here, as it has a highly lobed crenulate-type chloroplast and bear multiple pyrenoids per chloroplast (Fig. 1). Finally, in clade A, *T. decolorans* is the species with unique pyrenoid and chloroplast types, as mentioned above.

In contrast to the great diversity identified in clade A, clade S and clade C includes species (so far analyzed) with only one chloroplast architecture (Table 2). Species in clade C bear the corticola-type pyrenoid and thin lobed chloroplast, while species in clade S have an impressa-type pyrenoid and shallowly lobed chloroplast. However, some degree of diversity in the number of pyrenoids per chloroplast was observed within clade S (Table 2). In clade S, we also analyzed for the first time pyrenoid ultrastructure and chloroplast morphology for two newly recognized species-level lineages, i.e., *Trebouxia* sp. S08 [6] and the recently described species *T. australis* (S02) [28], which were recognized to bear both the impressa-type pyrenoid and shallowly lobed chloroplast. Species in clade I have an impressa pyrenoid type and shallowly lobed chloroplast, with the exception of *T. anticipata* which is characterized by the unique combination of the anticipata-type pyrenoid and the curly lobed chloroplast.

Observing chloroplast architecture in light of the phylogenetic delimitation may let us hypothesize on the evolution of this organelle within *Trebouxia.* However, the high degree of chloroplast diversity encountered within clade A in comparison with the low diversity recovered in clades C, I and S may be biased by the availability of cultured species-level lineages. Indeed, too few species-level lineages from clades C, I and S have been isolated in cultures so far -and these are included in the present analysis- and future analyses in these groups may uncover additional, unexpected diversity.

While the characterization of pyrenoid ultrastructure and chloroplast morphology was performed following a standardized axenic growth in culture, it remains to be seen whether and to what extent a certain diversity of these traits would be generated by changes in the algal metabolism. Also, if this functional diversity were potentially the results of these changes, we still would need to explore whether it involves important events in lichen biology and ecology, such as photobiont switching, intrathalline microalgal diversity and mycobiont-photobiont selectivity. Under another point of view, the diversity of the morpho-anatomical traits of the chloroplasts may translate into diversity of phycobiont functions and may lead to an improved capacity of the lichen to withstand environmental changes [57].

Our analyses highlight how chloroplast architecture proves to be a crucial tool to assess microalgal diversity for a reliable integrative taxonomy in lichen photobionts. Morphological and anatomical characters have not yet been analyzed for the majority of the recognized species-level lineages in *Trebouxia,* and future studies could potentially reveal either new type of pyrenoid ultrastructure and chloroplast morphology or new combination of the types here described. Furthermore, it will be crucial to correlate these in vitro traits with those exhibited in the symbiotic state within the lichen thalli.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102561.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

CRediT authorship contribution statement

CB, LM, PC and EB concepted and designed the study, CB, LM and SC collected and assembled the data, CB, LM analyzed and interpreted the data, CB, LM and SL drafted the article, EV and PC managed the funding, the administrative technical and logistic support.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Funding for this study was provided by the Prometeo Excellence in Research Program (PROMETEO/2017/039 and PROMETEO/2021/005, Generalitat Valenciana, Spain). Bordenave, C. D. received funding from a postdoctoral grant of the Valencian Government and the European Social Fund (APOSTD19).

Patricia Moya and Arantxa Molins (UVEG) are thanked for their support in the lab work and maintenance of the cultured strains.

References

[1] P. Škaloud, Y. Němcová, J. Pytela, N.I. Bogdanov, C. Bock, S.H. Pickinpaugh, Planktochlorella nurekis, gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid green alga carrying significant biotechnological potential, Fottea 14 (2014) 53–62.

[2] T. Darienko, L. Gustavs, A. Eggert, W. Wolf, T. Pröschold, Evaluating the species boundaries of green microalgae (Coccomyxa, tebouxiophyceae, Chlorophyta) using integrative taxonomy and DNA barcoding with further implications for the species identification in environmental samples, PLoS ONE 10 (2015), e0127838.

[3] C. Lemieux, C. Otis, M. Urmel, Chloroplast phylogenomic analysis resolves deep-level relationships within the green algal class Trebouxiophyceae, BMC Evol. Biol. 14 (2014) 211.

[4] S. Ma, V.A.R. Huss, D. Tan, X. Sun, J. Chen, Y. Xie, J. Zhang, A novel species in the genus heveochlorella (Trebouxiophyceae, Chlorophyta) witnesses the evolution from an epiphytic into an endophytic lifestyle in tree-dwelling green algae, Eur. J. Phycol. 48 (2013) 200–209.

[5] M.P. Nelsen, R. Lücking, C.K. Boyce, H.T. Lumbsch, R.H. Ree, The macroevolutionary dynamics of symbiotic and phenotypic diversification in lichens, Proc. Natl. Acad. Sci. 117 (2020) 21495–21503.

[6] L. Muggia, M.P. Nelsen, P.M. Kirika, E. Barreno, A. Beck, , Trebouxia working group, H. Lindgren, H.T. Lumbsch, S.D. Leavitt, Formally described species woefully underrepresent phylogenetic diversity in the common lichen photobiont genus Trebouxia (Trebouxiophyceae, Chlorophyta): an impetus for developing an integrated taxonomy, Mol. Phylogenet. Evol. 149 (2020), 106821.

[7] T. Friedl, Systematik und Biologie von Trebouxia (Microthamniales, Chlorophyta) als Phycobiont der Parmeliaceae (lichenisierte Ascomyceten), Chemie und Geowissenschaften der Universität Bayreuth, 1989. Dissertation zur Erlangung des Doktorgrades der Fakultät Biologie.

[8] T. Friedl, Comparative ultrastructure of pyrenoids in trebouxia (Microthamniales, Chlorophyta), Plant Syst. Evol. 164 (1989) 145–159, https://doi.org/10.1007/BF00940435.

[9] L. Muggia, S. Leavitt, E. Barreno, The hidden diversity of lichenized trebouxiophyceae (Chlorophyta), Phycologia 57 (2018) 503–524, https://doi.org/10.2216/17-134.1.

[10] P. Škaloud, J. Steinová, T. Řídká, L. Vančurová, O. Peksa, Assembling the challenging puzzle of algal biodiversity: species delimitation within the genus asterochloris (Trebouxiophyceae, Chlorophyta), J. Phycol. 51 (2015) 507–527, https://doi.org/10.1111/jpy.12295.

[11] E. Tscherman-Woess, The algal partner, in: M. Galun (Ed.), CRC Handbook of Lichenology Volume I, CRC Press Inc, Boca Raton, 1988, pp. 39–92.

[12] D. Hawksworth, M. Grube, Lichens redefined as complex ecosystems, New Phytol. 227 (2020) 1281–1283.

[13] R. Honegger, The symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts, in: B. Hock (Ed.), Fungal Associations, 2nd Edition The Mycota IX, Springer-Verlag Berlin, Heidelberg, 2012, pp. 287–339.

[14] L.M. Casano, E.M. del Campo, F.J. Garcìa-Breijo, J. Reig-Arminana, F. Gasulla, A. Del Hoyo, A. Guéra, E. Barreno, Two trebouxia algae with different physiological performances are ever present in lichen thalli of Ramalina farinacea. Coexistence versus competition? Environ. Microbiol. 13 (2011) 806–818.

[15] P. Moya, S. Chiva, A. Molins, I. Garrido-Benavent, E. Barreno, Unravelling the symbiotic microalgal diversity in Buellia zoharyi (lichenized Ascomycota) from the Iberian Peninsula and Balearic Islands using DNA metabarcoding, Diversity 13 (2021) 220, https://doi.org/10.3390/d13060220.

[16] M.P. Nelsen, S.D. Leavitt, K. Heller, L. Muggia, H.T. Lumbsch, Macroecological diversification and convergence in a clade of keystone symbionts, FEMS Microbiol. Ecol. 97 (2021), fiab072 (in press).

[17] S.D. Leavitt, E. Kraichak, J. Vondrak, M.P. Nelsen, M. Sohrabi, S. Perez-Ortega, L. St Clair, H.T. Lumbsch, Cryptic diversity and symbiont interactions in rock-posy lichens, Mol. Phylogenet. Evol. 99 (2015) 261–274.

[18] A. Beck, Selektivität der Symbionten schwermetalltoleranter Flechten. VIII, 194 p. Doctoral thesis, Universität München, 2002. ISBN 3-9808102-0-8.

[19] G. Helms, in: Taxonomy and Symbiosis in Associations of Physciaceae and Trebouxia, Universität Göttingen, 2003, p. 158. Doctoral Thesis.

[20] L. Muggia, S. Pérez-Ortega, T. Kopun, G. Zellnig, M. Grube, Photobiont selectivity leads to ecological tolerance and evolutionary divergence in a polymorphic complex of lichenized fungi, Ann. Bot. 114 (2014) 463–475.

[21] M. Xu, H. De Boer, E.S. Olafsdottir, S. Omarsdottir, S. Heidmarsson, Phylogenetic diversity of the lichenized algal genus trebouxia (Trebouxiophyceae, Chlorophyta): a new lineage and novel insights from fungal-algal association patterns of icelandic cetrarioid lichens (Parmeliaceae, Ascomycota), Bot. J. Linn. Soc. 194 (2020) 460–468, https://doi.org/10.1093/botlinnean/boaa050.

[22] A. Molins, P. Moya, F.J. García-Breijo, J. Reig-Armiñana, E. Barreno, Assessing lichen microalgal diversity by a multi-tool approach: isolation, sanger sequencing, HTS and ultrastructural correlations, Lichenologist 50 (2018) 123–138.

[23] P. Moya, P. Škaloud, S. Chiva, F.J. Garcia-Breijo, J. Reig-Arminana, L. Vančurova, E. Barreno, Molecular phylogeny and ultrastructure of the lichen microalga asterochloris mediterranea sp. nov. from Mediterranean and Canary Islands ecosystems, Int. J. Syst. Evol. Microbiol. 65 (2015) 1838–1854.

[24] H. Ettl, G. Gärtner, Über die bedeutung der cytologie für die algentaxonomie, dargestellt an trebouxia (Chlorellales, Chlorophyceae), Plant Syst. Evol. 148 (1) (1984) 135–147.

[25] H. Ettl, G. Gärtner, in: Syllabus der Boden-, Luft- und Flechtenalgen; Gärtner 1985: Die Gattung Trebouxia Puymaly (Chlorellales, Chlorophyceae). Algological Studies, Gustav Fischer Verlag, Stuttgart, 1995, p. 721.

[26] G. Gärtner, E. Ingolic, Zur morphologie und systematik des trebouxia phycobionten im thallus von Usnea longissima (Lecanorales), Plant Syst. Evol. 158 (1988) 225–234.

[27] G. Gärtner, E. Ingolic, Problems in the identification of lichen photobionts, Sauteria 9 (1998) 373–380.

[28] A. Voytsekhovich, A. Beck, Lichen photobionts of the rocky outcrops of karadag massif (Crimean Peninsula), Symbiosis 68 (2016) 9–24.

[29] P.A. Archibald, Trebouxia de pulmaly (Chlorophyceae, Chlorococcales) and pseudotrebouxia gen. nov. (Chlorophyceae, Chlorosarcinales), Phycologia 14 (1975) 125–137.

[30] M. Meyer, H. Griffiths, Origins and diversity of eukaryotic CO2-concentrating mechanisms: lessons for the future, J. Exp. Bot. 64 (2013) 769–786, https://doi.org/10.1093/jxb/ers390.

[31] M.T. Meyer, A.K. Itakura, W. Patena, L. Wang, S. He, T. Emrich-Mills, C.S. Lau, G. Yates, L.C.M. Mackinder, M.C. Jonikas, in: Assembly of the Algal CO2-Fixing Organelle, the Pyrenoid, is Guided by a Rubisco-binding Motif, bioRxiv, 2020, pp. 1–11, https://doi.org/10.1101/2020.08.16.252858.

[32] N. Atkinson, Y. Mao, K.X. Chan, A.J. McCormick, Condensation of rubisco into a proto-pyrenoid in higher plant chloroplasts, Nat. Commun. 11 (2020), https://doi.org/10.1038/s41467-020-20132-0.

[33] A. Mukherjee, J.V. Moroney, How protein - protein interactions contribute to pyrenoid formation in chlamydomonas, J. Exp. Bot. 70 (2019) 5033–5035, https://doi.org/10.1093/jxb/erz299.

[34] F. Martínez-Alberola, Caracterización genómica del microalga Trebouxia sp. TR9 aislada del liquen Ramalina farinacea (L.) Ach. mediante secuenciación masiva, PhD Dissertation, Universitat de València, 2015 (2015), http://roderic.uv.es/handle/10550/48824.

[35] S. Català, E.M. del Campo, E. Barreno, F.J. García-Breijo, J. Reig-Armiñana, L. M. Casano, Coordinated ultrastructural and phylogenomic shed light on the hidden phycobiont diversity of the trebouxia microalgae in Ramalina fraxinea, Mol. Phylogenet. Evol. 94 (2016) 765–777.

[36] H.W. Bischoff, H.C. Bold, Phycological Studies. IV. Some Soil Algae From Enchanted Rock and Related Algal Species, University of Texas Publications, 6318, Austin, Texas, 1963.

[37] H.C. Bold, B.C. Parker, Some supplementary attributes in the classification of chlorococcum species, Arch. Mikrobiol. 42 (1962) 267–288.

[38] L. Muggia, S.D. Leavitt, E. Barreno, Report of the meeting of the Trebouxia-working group, Trieste, Italy, 2016, Int. Lichenological Newsl. 49 (2016) 35–37.

[39] M. Bačkor, D. Fahselt, Cellulose-acetate disks as novel substrate for the resynthesis and culture of lichens, Bryologist 106 (2003) 439–442.

[40] S. Kroken, J.W. Taylor, Phylogenetic species, reproductive mode, and specificity of the green alga trebouxia forming lichens with the fungal genus letharia, Bryologist 103 (2000) 645–660.

[41] L. Lindblom, S. Ekman, Genetic variation and population differentiation in the lichen-forming ascomycete Xanthoria parietina on the island storfosna, Central Norway, Mol. Ecol. 15 (2006) 1545–1559.

[42] K. Katoh, G. Asimenos, H. Toh, Multiple alignment of DNA sequences with MAFFT, Methods Mol. Biol. 537 (2009) 39–64.

[43] R. Mason-Gamer, E. Kellogg, Testing for phylogenetic conflict among molecular dataset in the tribe triticeae (Gramiae), Syst. Biol. 45 (1996) 524–545.

[44] V. Reeb, F. Lutzoni, C. Roux, Contribution of RPB2 to multilocus phylogenetic studies of the euascomycetes (Pezizomycotina, Fungi) with special emphasis on the lichen-forming acarosporaceae and evolution of polyspory, Mol. Phylogenet. Evol. 32 (2004) 1036–1060.

[45] A. Stamatakis, RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies, -, Bioinformatics 30 (2014) 1312–1313, http://bioinformatics.oxfordjournals.org/.

[46] R.D.M. Page, TREEVIEW: an application to display phylogenetic trees on personal computers, Comput. Appl. Biosci. 12 (1996) 357–358.

[47] A. Molins, P. Moya, F.J. García-Breijo, J. Reig-Armiñana, E. Barreno, Molecular and morphological diversity of trebouxia microalgae in sphaerothallioid circinaria spp. lichens1, J. Phycol. 54 (2018) 494–504, https://doi.org/10.1111/jpy.12751.

[48] P. Moya, A. Molins, F. Martínez-Alberola, L. Muggia, E. Barreno, Unexpected associated microalgal diversity in the lichen Ramalina farinacea is uncovered by pyrosequencing analyses, PLoS One 12 (2017), e0175091.

[49] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682, https://doi.org/10.1038/nmeth.2019.

[50] L. Vančurová, O. Peksa, Y. Němcová, P. Škaloud, Vulcanochloris (Trebouxiales, Trebouxiophyceae), a new genus of lichen photobiont from La Palma, Canary Islands, Spain, Phytotaxa 219 (2015) 118–132, https://doi.org/10.11646/phytotaxa.219.2.2.

[51] P. Moya, S. Chiva, A. Molins, I. Jadrná, P. Škaloud, O. Peksa, E. Barreno, Myrmecia israeliensis as the primary symbiotic microalga in squamulose lichens growing in european and Canary Island terricolous communities, Fottea 18 (2018) 72–85.

[52] M. Bačkor, P. Váczi, Copper tolerance in the lichen photobiont Trebouxia erici (Chlorophyta), Environ. Exp. Bot. 48 (2002) 11–20.

[53] S.J. Goldsmith, M.A. Thomas, C. Gries, A new technique for photobiont culturing and manipulation, Lichenologist 29 (1997) 559–569.

[54] A. del Hoyo, R. Álvarez, E.M. del Campo, F. Gasulla, E. Barreno, L.M. Casano, Oxidative stress induces distinct physiological responses in the two trebouxia phycobionts of the lichen Ramalina farinacea, Ann. Bot. 107 (2011) 109–118, https://doi.org/10.1093/aob/mcq206.

[55] F. Gasulla, P.G. de Nova, A. Esteban-Carrasco, J.M. Zapata, E. Barreno, A. Guéra, Dehydration rate and time of desiccation affect recovery of the lichenic algae Trebouxia erici: alternative and classical protective mechanisms, Planta 231 (2009) 195–208, https://doi.org/10.1007/s00425-009-1019-y.

[56] E. Hinojosa-Vidal, F. Marco, F. Martínez-Alberola, F.J. Escaray, F.J. García-Breijo, J. Reig-Armiñana, P. Carrasco, E. Barreno, Characterization of the responses to saline stress in the symbiotic green microalga trebouxia sp. TR9, Planta 248 (2018) 1473–1486, https://doi.org/10.1007/s00425-018-2993-8.

[57] F. Martínez-Alberola, E. Barreno, L.M. Casano, F. Gasulla, A. Molins, P. Moya, M. González-Hourcade, E.M. Del Campo, The chloroplast genome of the lichen-symbiont microalga trebouxia sp. TR9 (Trebouxiophyceae, Chlorophyta) shows short inverted repeats with a single gene and loss of the RPS4 gene, which is encoded by the nucleus, J. Phycol. 56 (2020) 170–184.

[58] A. Molins, P. Moya, L. Muggia, E. Barreno, Thallus growth stage and geographic origin shape Microalgal diversity in the lichen Ramalina farinacea, J. Phycol. (2021), https://doi.org/10.1111/jpy.13140. Dissertation zur Erlangung des Doktorgrades der Fakultät Biologie.