The Lichenologist (2024), **56**, 107–119
doi:10.1017/S0024282924000069

CAMBRIDGE UNIVERSITY PRESS

Standard Paper

Chloroidium phycobionts (*Watanabeales*, *Trebouxiophyceae*) partner with lecanoralean mycobionts in foliicolous lichen communities of Tenerife (Canary Islands) and Navarra (Iberian Peninsula), Spain

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Abstract

While the diversity of foliicolous lichen-forming fungi has been explored in substantial depth, relatively little attention has been paid to their algal symbionts. We studied the unicellular green phycobionts of the lecanoralean lichens *Bacidina* (*Ramalinaceae*), *Byssoloma*, *Fellhanera* and *Tapellaria* (*Pilocarpaceae*) and graphidalean *Gyalectidium* (*Gomphillaceae*) from two extratropical foliicolous communities in continental Spain and the Canary Islands. We examined the pyrenoids of algal symbionts within thalli using TEM, and obtained several algal nrSSU and *rbcL* sequences from whole thalli, and also from cultures isolated from some of these lichens. Pyrenoid structure and molecular sequence data provided support for recognizing *Chloroidium* (*Watanabeales*, *Trebouxiophyceae*) as phycobiont in thalli of *Byssoloma subdiscordans* and *Fellhanera bouteillei* (*Pilocarpaceae*) in both communities. *Bacidina apiahica* (*Ramalinaceae*) and *Tapellaria epiphylla* (*Pilocarpaceae*) likewise appeared to partner with *Chloroidium* based on the presence of the same pyrenoid type, although we were able to obtain a phycobiont sequence only from a culture isolate of the latter. These results contrast with those obtained previously from a foliicolous lichen community in southern Florida, which revealed only strains of *Heveochlorella* (*Jaagichlorella*) as phycobiont of foliicolous *Pilocarpaceae* and *Gomphillaceae*. On the other hand, the pyrenoid we observed in the phycobionts associated with *Gyalectidium setiferum* and *G. minus* corresponded to that of *Heveochlorella* (*Jaagichlorella*). However, the poor quality of the phycobiont sequence data obtained from *G. minus*, probably due to the presence of epibiontic algae, could not provide additional perspective on the pyrenoid structure observations. Nonetheless, clear differences in pyrenoid ultrastructure can allow *Chloroidium* and *Heveochlorella* phycobionts to be distinguished from each other in TEM. Our results indicate a greater diversity of unicellular green-algal symbionts in foliicolous communities from Spain than previously observed in other geographical areas, and suggest that further studies focused on symbiont pairing in these communities might reveal distinctive and varied patterns of phycobiont preference.

Keywords: *Heveochlorella*; *Jaagichlorella*; photobiont; phycobiont; symbiosis

(Accepted 17 January 2024; first published online 26 March 2024)

Introduction

The leaf-colonizing (foliicolous) lichens of the humid tropics and subtropics are specialists whose distinctive features are related to the unique conditions associated with their microhabitat and their ephemeral, living substratum (Lücking 2001). Significant attention has been given to the lichen-forming fungi of foliicolous communities (Santesson 1952; Lücking 2008), particularly in the Neotropics, but their algal symbionts remain little studied. The filamentous *Trentepohliaceae* (*Trentepohlia*, *Phycopeltis*, *Cephaleuros*) are clearly important phycobionts in many of these communities (Lücking 2008). However, the two principal families of foliicolous lichen-forming fungi, the *Pilocarpaceae* (*Lecanorales*) and the *Gomphillaceae* (*Graphidales*), associate with unicellular green algae whose identities have only recently been subjected to any scrutiny. In a foliicolous community of southern Florida, all sampled species of both *Pilocarpaceae* and *Gomphillaceae* were found to partner with the alga *Heveochlorella* (Sanders *et al.* 2016). This recently described trebouxiophycean genus first contained non-lichenized bark colonists from South-East Asia (Zhang *et al.* 2008; Ma *et al.* 2013) but has since been found to include lichen symbionts worldwide (Dal Grande *et al.* 2014; Sanders *et al.* 2016; Lindgren *et al.* 2020). *Heveochlorella* is one of *c.* 10 rather new genera within the recently recognized order *Watanabeales*, in which many new taxa are being described (Darienko *et al.* 2018; Li *et al.* 2021). Darienko & Pröschold (2019) combine *Heveochlorella* and the related *Heterochlorella* (Neustupa *et al.* 2009) into the genus *Jaagichlorella*. We accept their arguments for uniting the two genera but continue to refer to *Heveochlorella* for the purposes of the present study, since a consistent pyrenoid type is associated with those taxa ascribed to

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Cite this article: Sanders WB, de los Ríos A and Pérez-Ortega S (2024) *Chloroidium* phycobionts (*Watanabeales*, *Trebouxiophyceae*) partner with lecanoralean mycobionts in foliicolous lichen communities of Tenerife (Canary Islands) and Navarra (Iberian Peninsula), Spain. *Lichenologist* **56**, 107–119. https://doi.org/10.1017/S0024282924000069

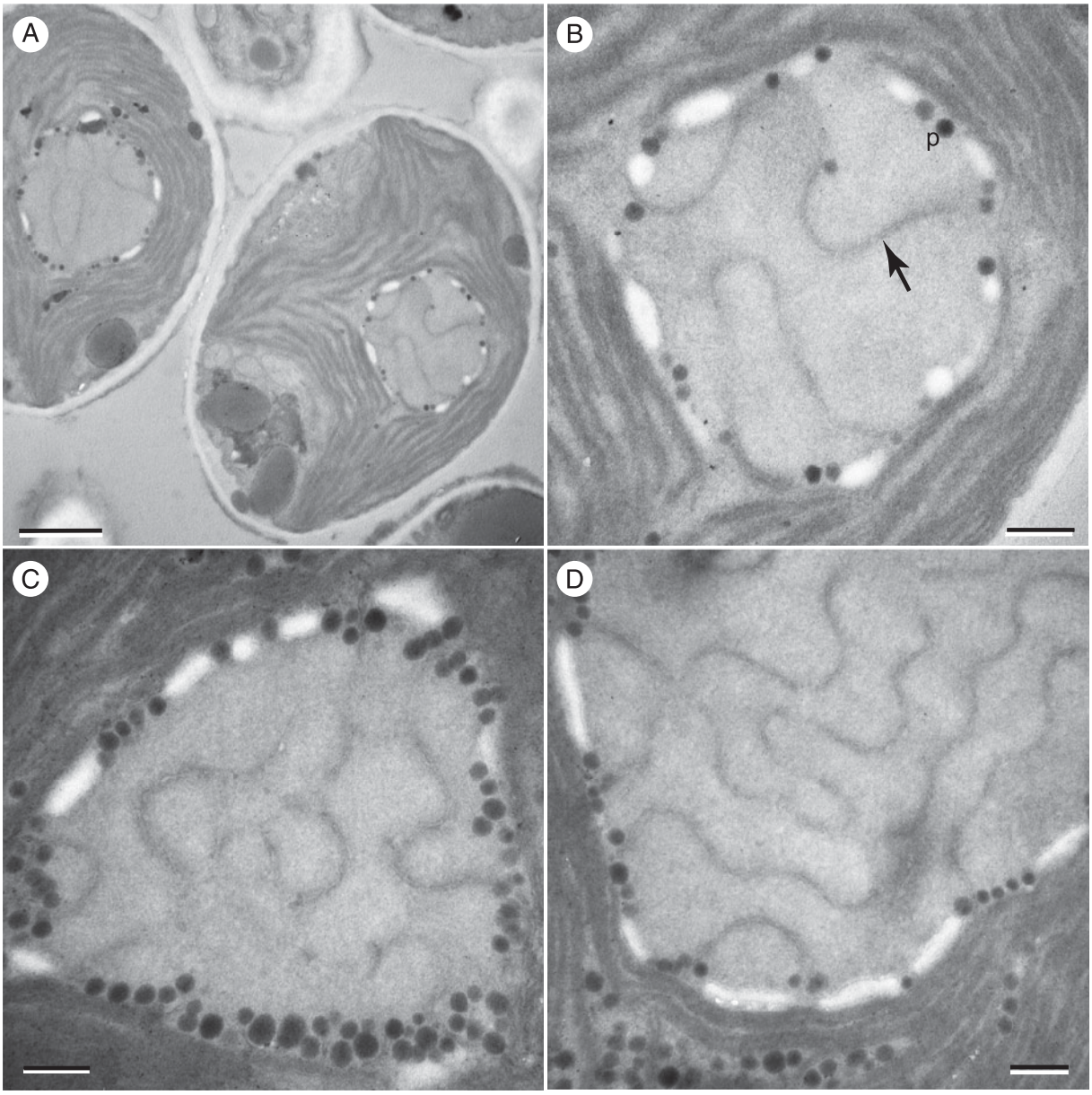

Figure 1. TEM images of pyrenoid of phycobiont associated with two foliicolous lichens from Navarra. Arrow indicates sinuous thylakoid traversing the pyrenoid; p = pyrenoglobule. A & B, *Byssoloma subdiscordans*. C & D, *Fellhanera bouteillei*. Scales: A = 1 µm; B–D = 250 nm.

Heveochlorella, whereas the type culture of *Heterochlorella* apparently has a pyrenoid structure (Neustupa *et al.* 2009, figs. 26 & 27) completely different from that reported in all other strains of both genera examined with TEM, an oddity that deserves further investigation. For the type species of *Jaagichlorella* (*J. geometrica*), a pyrenoid structure is not known.

Although *Heveochlorella* was the only phycobiont genus found in *Gomphillaceae* and *Pilocarpaceae* from the foliicolous community studied in Florida, two Brazilian specimens of *Gomphillaceae* collected from bark substratum partnered with *Chloroidium* (*Watanabeales*), while the phycobiont of one foliicolous member of *Pilocarpaceae* from Panama fell within an unidentified clade close to *Chloroidium* (Sanders *et al.* 2016). Those results suggested that foliicolous members of the two fungal families in question might well associate with a more diverse range of phycobionts in other localities. Since foliicolous members of *Pilocarpaceae* and *Gomphillaceae* occur throughout the humid tropics, extending into the subtropics and even some habitats within temperate latitudes (van den Boom 2020), their relationships with algal symbionts may be of considerable importance in understanding their ecological success. In the present study, we examine thallus phycobionts in two rather isolated extratropical communities of foliicolous lichens in Macaronesian and continental Spain.

Methods and Materials

Sampling, isolation and culture of photobionts

Foliicolous lichens were collected with their leaf substratum from two localities in Spain: the Foz de Arbayún gorge in Navarra (42.68°N, 1.19°W, *c.* 460 m; *Gyalectidium setiferum* Vězda & Sérus., *Fellhanera bouteillei* (Desm.) Vězda and *Byssoloma*

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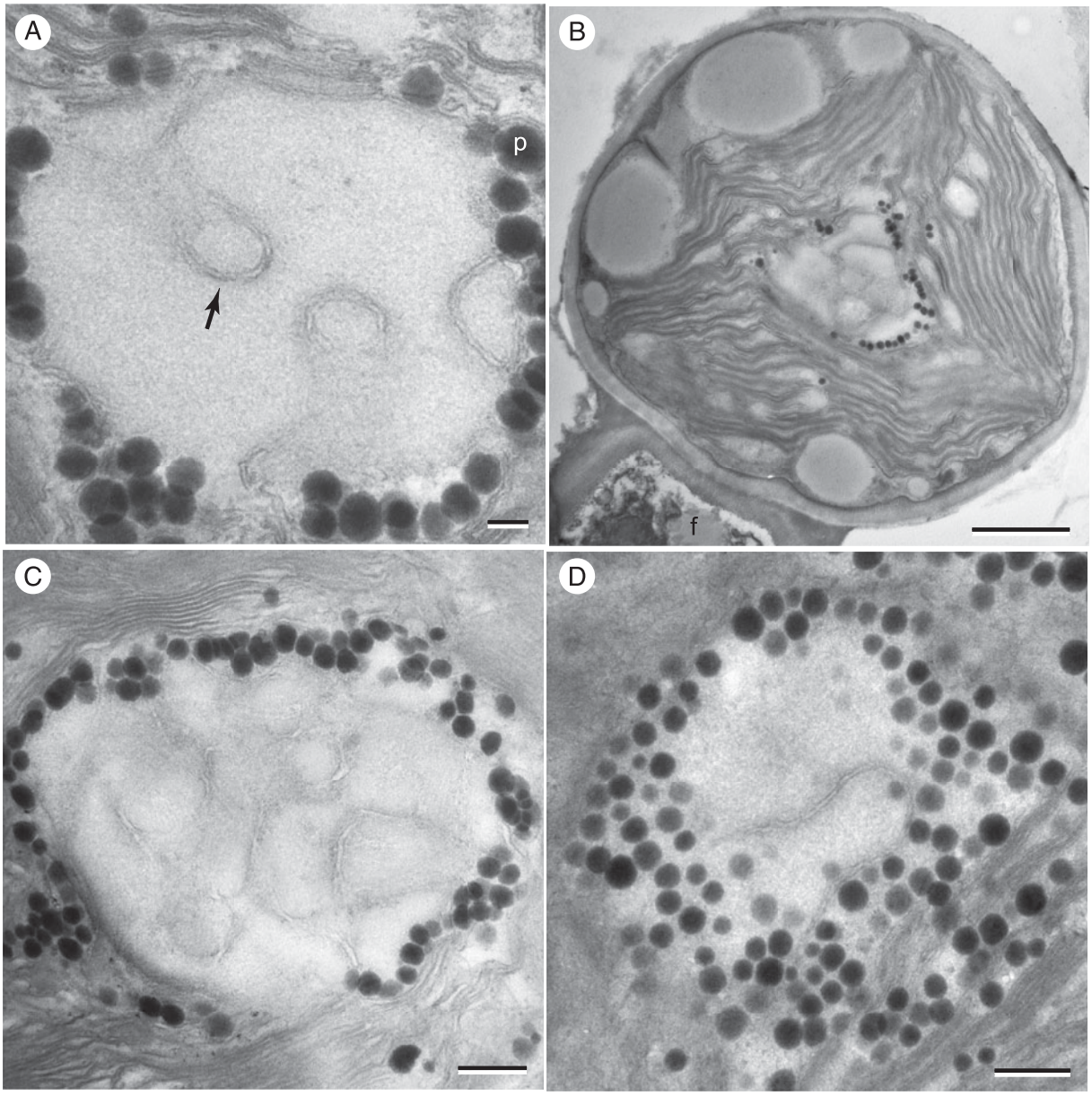

Figure 2. TEM images of pyrenoid of phycobiont associated with three foliicolous lichens from Tenerife. A, *Bacidina apiahica*; arrow = thylakoid penetrating pyrenoid; p = pyrenoglobule. B & C, *Byssoloma subdiscordans*; f = contacting fungal cell. D, *Fellhanera bouteillei*. Scales: A = 100 nm; B = 1 µm; C & D = 250 nm.

subdiscordans (Nyl.) P. James), and the Anaga laurisilva forest in Tenerife, Canary Islands (28.53°–28.58°N, 16.17°–16.18°W, 100–900 m; *Gyalectidium minus* Sérus., *Bacidina apiahica* (Müll. Arg.) Vězda, *Byssoloma kakouettae* (Sérus.) Lücking & Sérus., *B. subdiscordans* and *Fellhanera bouteillei*). *Gyalectidium* spp. are members of *Gomphillaceae* (*Graphidales*), while the remaining genera belong in *Pilocarpaceae* (*Byssoloma*, *Fellhanera*, *Tapellaria*) and *Ramalinaceae* (*Bacidina*) within the *Lecanorales*. Identifications were made with reference to Lücking (2008). Foliicolous thalli were peeled from drying leaves with sterile forceps or scraped with a sterile razor blade and placed in a centrifuge tube for direct PCR or DNA extraction. Several thalli or thallus areolae from the same or neighbouring leaves were often included within the same sample to ensure sufficient material for analysis, usually judged as enough when clearly visible in the tube with the naked eye. For culturing, a small thallus or thallus areola was peeled from the leaf and pressed underside down onto 1× BBM agar. Special care was taken to avoid thalli with an obvious presence of epiphytic algae or lichenicolous fungi. Culture plates were maintained at room temperature in the laboratory near to windows but without exposure to direct sunlight. Other thalli were cut out with thin strips of their leaf substratum using a razor blade and processed for TEM as indicated below.

TEM processing

Fresh material was misted with distilled water a few hours prior to hand-sectioning with a thin razor blade. Sections were placed immediately into tubes with 3% glutaraldehyde in phosphate buffer for *c.* 3 h, then washed three times in buffer, post-fixed with 1% osmium tetroxide in phosphate buffer for *c.* 5 h, washed

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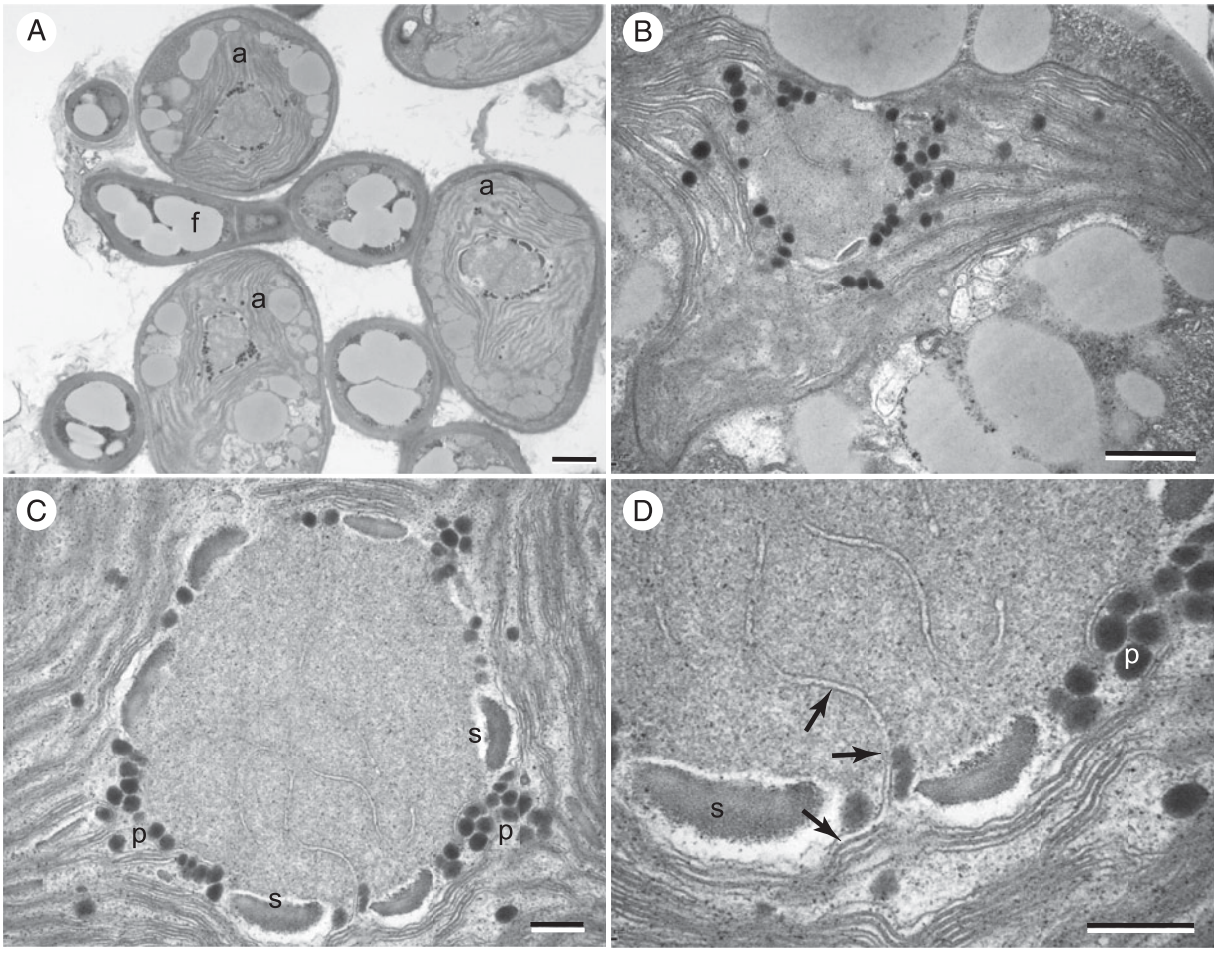

Figure 3. A–D, TEM images of phycobiont within the thallus of *Tapellaria epiphylla* from Tenerife. Arrows indicate continuity of pyrenoid-penetrating thylakoids with stacked thylakoids outside pyrenoid; a = algal symbiont; f = fungal symbiont; p = plastoglobule; s = starch. Scales: A = 1 µm; B = 500 nm; C & D = 250 nm.

again, and dehydrated in a graded ethanol series. Specimens were then infiltrated with Spurr's low viscosity resin (initially diluted with propylene oxide) and polymerized (de los Ríos & Ascaso 2002). Specimen blocks (1–2 per lichen species in each community) were sectioned with an Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and imaged with a JEOL transmission electron microscope at the Centro Nacional de Biotecnología (CNB-CSIC).

DNA extraction and amplification

Thallus fragments in microcentrifuge tubes were stored at −80 °C and, after 1 h of freezing, were pulverized using a Qiagen TissueLyser II and glass beads. DNA was extracted using E.Z.N.A.® Forensic DNA Kit (Omega Bio-Tek) according to the manufacturer's instructions, with final eluting of extracted DNA in water (50 µl). DNA from phycobiont cultures was extracted using a SpeedTools Tissue DNA extraction kit according to the manufacturer's protocol. A fragment of the nuclear ribosomal small subunit (nrSSU) gene was amplified using the primer pair 232F (Thüs *et al.* 2011) and SR7R (R. Vilgalys, unpublished data; http://www.botany.duke.edu/fungi/mycolab). A fragment of the gene that encodes the large subunit of ribulose-1,5 bisphosphate carboxylase (*rbcL*) was amplified using the primer pair RH1 (Manhart 1994) and rbcL 530R (Verbruggen *et al.* 2009). Amplification reactions using DNA extracted from the thallus were prepared for a 15 µl final volume containing 7.5 µl of MyTaq™ Red Mix (Bioline), 0.5 µl of each of the primers at 10 µM, 5.5 µl of H_2O, and 1 µl of template. Reactions using DNA extracted from cultures were prepared for a 25 µl final volume using GE Healthcare Illustra™ PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences), 0.5 µl of each of the primers at 10 µM, 21 µl of H_2O, and 3 µl of DNA template. PCR conditions for amplification of the nrSSU and *rbcL* were as follows: 5 min at 94 °C; 34 cycles of 45 s at 95 °C, 45 s at 57 °C (nrSSU) or 50 °C (*rbcL*), 1 min 30 s at 72 °C, and a final extension step of 5 min at 72 °C. Due to a lack of success in amplifying the selected regions from DNA extractions, we employed a different strategy using direct PCR. Direct PCR reactions were carried out using KAPA3 G Plant PCR Kit (KAPA Biosystems), which contains the KAPA3 G Plant DNA Polymerase (2.5 U μl^{-1}) and KAPA Plant PCR Buffer with dNTPs (2×, with 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP at 1×), following the manufacturer's instructions. PCR reactions were prepared in a 50 µl final volume containing the tiny fragment of lichen thallus (*c.* 1 mm^2), 25 µl of the KAPA Plant PCR Buffer, 1.5 µl of each primer at 10 µM and 0.4 µl of the KAPA3 G Plant DNA Polymerase, and sterile water to complete the volume. The following cycling protocol was used: 3 min at 95 °C, 35 cycles of 20 s at 95 °C, 15 s at 57 °C (nrSSU) or 50 °C (*rbcL*), 30 s at 72 °C, and a final extension

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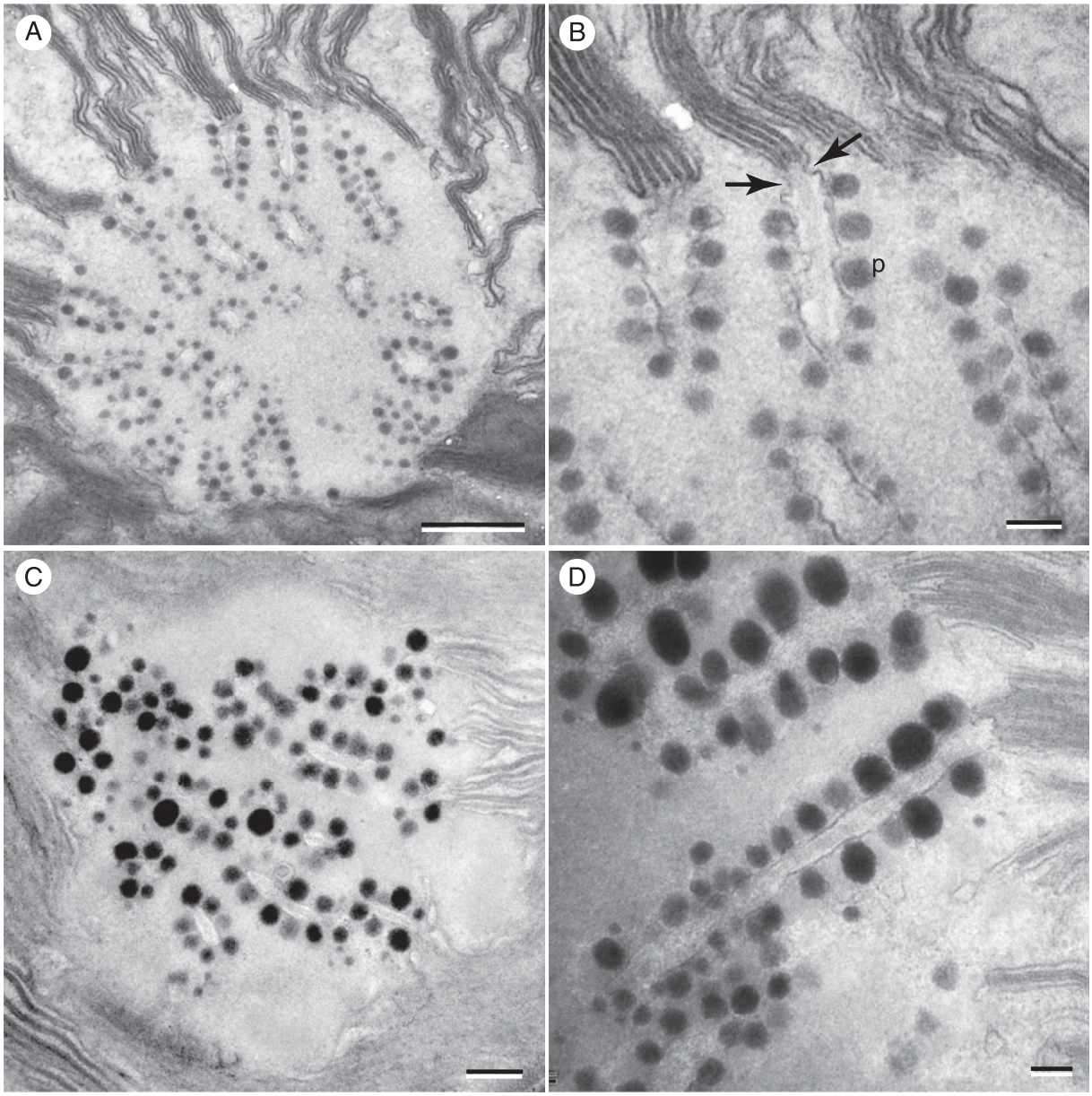

Figure 4. TEM images of phycobiont pyrenoids in *Gyalectidium*. A & B, *G. setiferum* (Navarra). C & D, *G. minus* (Tenerife). B, tubule arising from outer membranes of thylakoid stack (arrowed); p = pyrenoglobule. Scales: A = 500 nm; B & D = 100 nm; C = 250 nm.

of 1 min at 72 °C. Both complementary strands were sequenced by Macrogen Inc. (Madrid, Spain) using the same primer set as for PCR amplification. Sequences were inspected and contigs assembled using Geneious Prime® v. 2020.0.3. Curated sequences were used as a template for BLAST searches (Altschul *et al.* 1997).

Phylogenetic analyses

A BLAST search was carried out in GenBank to ensure that the sequences obtained belonged to groups that included phycobionts rather than likely contaminants. For the *rbcL* sequences, all BLAST search results retrieved members of *Chloroidium*, so we compiled sequences from representative members of genera in the *Watanabeales* following Darienko & Pröschold (2019) and Li *et al.* (2021) (see Supplementary Material Table S1, available online). Members of *Diplosphaera*, *Stichococcus* and *Symbiochloris* were used as outgroup. Regarding nrSSU sequences, in addition to *Chloroidium*, we also obtained sequences from other genera of *Trebouxiophyceae*, so we compiled a larger dataset with data of representative genera of the group based on Li *et al.* (2021), focusing on the groups retrieved from the BLAST search (Supplementary Material Table S2, available online). Sequences from *Chlorophyceae* species *Oedogonium cardiacum*, *Chaetopeltis orbicularis* and *Floydiella terrestris* were used as outgroup.

Alignments for each locus were carried out using MAFFT v. 7.490 (Katoh *et al.* 2002) as implemented in the software Geneious Prime® v. 2022.2.2 (https://www.geneious.com) using default parameters. Alignments were visually inspected and introns were removed using Gblocks v. 0.91b (Castresana 2000) (at http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks) applying all the options available for the least stringent selection. The alignments were analyzed using maximum

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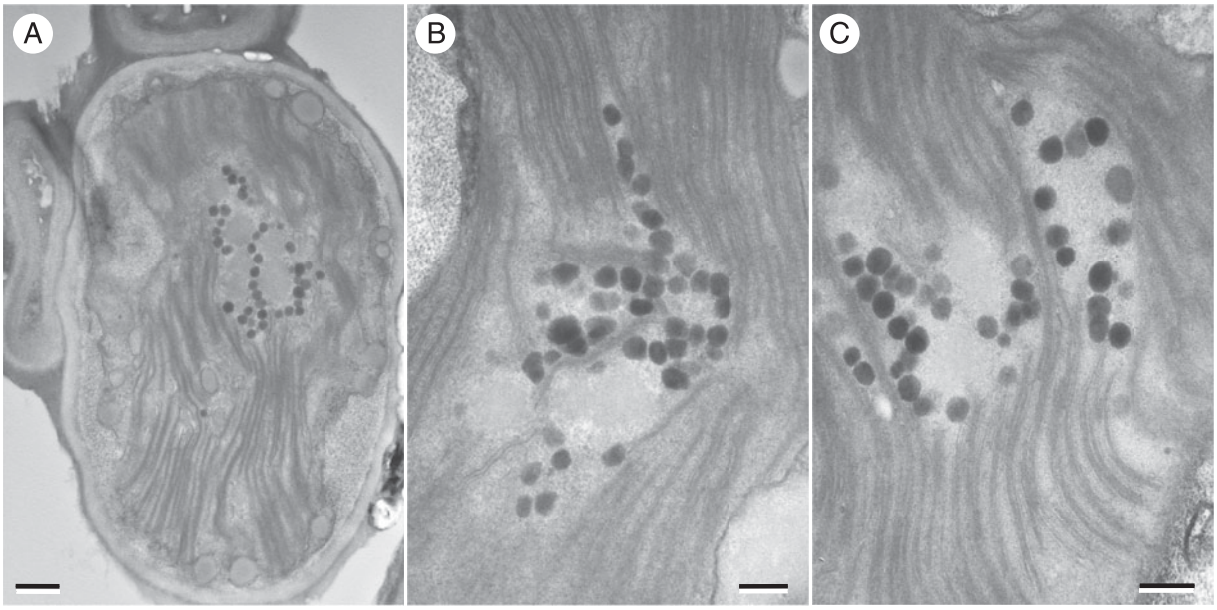

Figure 5. TEM images of phycobiont within the thallus of *Byssoloma leucoblepharum*. A, alga with contacting mycobiont cells. B & C, diffuse pyrenoidal areas with pyrenoglobuli. Scales: A = 500 nm; B & C = 250 nm.

likelihood (ML) phylogenetic inference methods. Analyses were performed in RAxML v. 8.2.11 (Stamatakis 2014) as implemented in Geneious Prime® v. 2022.2, using the GTRGAMMA substitution model. We conducted the search of the best-scoring ML tree and rapid bootstrapping with 1000 pseudoreplicates to evaluate nodal support in one single run. We considered supported nodes (depicted in bold in Figs 7 & 8) to be those with bootstrap values ≥ 70%. Trees were visualized using FigTree v. 1.4.4 (available at https://github.com/rambaut/figtree/releases) and Adobe Illustrator CS5 was used for artwork.

Results

Pyrenoid ultrastructure

TEM observations of phycobionts within foliicolous lichen thalli revealed three different pyrenoid types, two well-defined and one diffuse, present among algal symbionts of the two communities studied. The most common type was typically prominent, central, roughly round in section, and penetrated by one or a small number of slender, membranous plates that meandered sinuously through the pyrenoid, which they appeared to fully traverse while passing in and out of the plane of section (Figs 1–3). The penetrating membranes were continuous with chloroplast thylakoids outside the pyrenoid and each maintained the approximate width of a single thylakoid (Fig. 3D). Pyrenoglobuli were generally abundant and arranged at the outer periphery of the pyrenoid; they were not associated with the penetrating membranes or any part of the pyrenoid interior. Often, starch deposits were also seen at the pyrenoid periphery (Figs 1 & 3). This type of pyrenoid was observed in thalli of *Byssoloma subdiscordans* and *Fellhanera bouteillei* from both Navarra and Tenerife (Figs 1 & 2), as well as in those of *Bacidina apiahica* (Fig. 2A) and *Tapellaria epiphylla* (Müll. Arg.) R. Sant. (Fig. 3) from Tenerife.

The second type of pyrenoid observed was penetrated by membranous tubules arising from the outermost membranes of a stack of several thylakoids; the tubules often maintained the approximate width of the entire stack rather than that of a single thylakoid (Fig. 4A & B). The penetrating tubules were oriented centripetally, appearing in both longitudinal and transverse view; their trajectories appeared to end near the centre of the pyrenoid, with no individual tubule seen to fully traverse it (Fig. 4). Pyrenoglobuli lined the exterior surfaces of these penetrating tubules. Surrounding starch deposits were not seen. This type of pyrenoid was observed in *Gyalectidium setiferum* from Navarra and *G. minus* from Tenerife (Fig. 4).

A third, diffuse type of pyrenoid was represented by smallish, dilated areas of modest electron density between thylakoid stacks; they included pyrenoglobuli, often at the periphery but with no discernible regularity in arrangement (Figs 5, 6A & B). This poorly defined pyrenoid might be confused with peripheral, tangential sections of a larger, central pyrenoid that has pyrenoglobuli at its perimeter (e.g. Fig. 2D). However, examination of numerous sectioned phycobiont cells suggested that this was a distinct type. It was observed in phycobionts within thalli of *Byssoloma leucoblepharum* (Nyl.) Vain. (Fig. 5) and *B. kakouettae* (Fig. 6A & B) from Tenerife, although an algal culture isolated from thalli of *B. kakouettae* clearly showed the first type of pyrenoid described above (Fig. 6C & D).

Molecular sequencing

Many attempts to amplify selected regions of foliicolous phycobiont DNA resulted in sequences showing clear double or even triple peaks in parts of the electropherograms. Clean nrSSU (11) and *rbcL* (9) algal sequences were obtained from thalli of *Byssoloma subdiscordans* and *Fellhanera bouteillei* from both localities, and *B. leucoblepharum* and *Gyalectidium minus* from Tenerife. In addition, we obtained clean nrSSU (4) and *rbcL* (4) sequences from the phycobionts of *Byssoloma kakouettae*, *B. subdiscordans*, *Fellhanera bouteillei* and *Tapellaria epiphylla* from Tenerife isolated into culture (Table 1). We were unsuccessful in obtaining clean sequences from the thalli of *Bacidina apiahica*, *Byssoloma kakouettae* and *Tapellaria epiphylla*. All recovered *rcbL*

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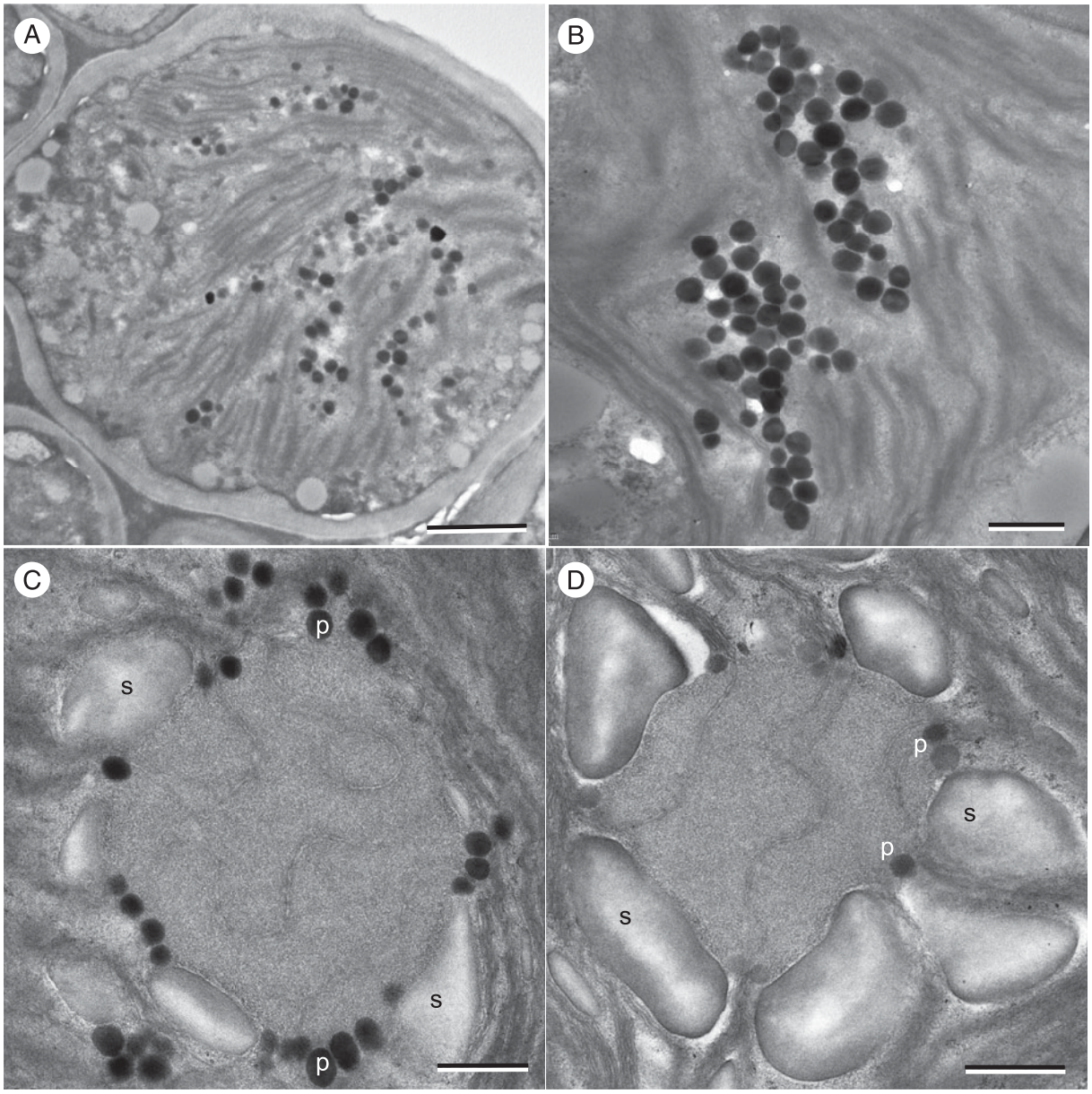

Figure 6. A & B, TEM images of phycobiont within the thallus of *Byssoloma kakouettae* (Tenerife), showing diffuse pyrenoidal areas. C & D, TEM images of pyrenoid in alga isolated into culture from the thallus of *Byssoloma kakouettae* (Tenerife). C, with numerous electron-dense pyrenoglobuli and some starch deposits at periphery. D, with large starch deposits and few pyrenoglobuli at periphery; p = pyrenoglobule; s = starch. Scales: A = 1 μm; B–D = 500 nm.

sequences, both from thalli and cultures from Tenerife and Navarra, formed a well-supported clade within the genus *Chloroidium*, sister to *C. angustoellipsoideum* although this relationship was not supported (Fig. 7). The sequences were identical except for Arb49 and Arb50 (both from *Fellhanera bouteillei* from Navarra) and E135 (*Byssoloma subdiscordans* from Tenerife), which differed in three positions. Regarding nrSSU, most of the obtained sequences formed two clades within *Chloroidium.* One clade contained all sequences obtained from lichen thalli (*Byssoloma subdiscordans* from Navarra and Tenerife, *Fellhanera bouteillei* from Navarra, and *Gyalectidium minus* from Tenerife) together with sequences of *C. ellipsoideum*, *C. lichenum* and *C. angustoellipsoideum* obtained from GenBank (Fig. 8). The sister group to this clade consisted of all sequences obtained from phycobionts in culture. In addition, two sequences from *Byssoloma leucoblepharum* (A31 and A32 from Tenerife) were found to be related to species of the genus *Symbiochloris*, while one sequence from *Fellhanera bouteillei* (Arb42 from Navarra) belonged to the genus *Trebouxia* and one sequence from *Byssoloma subdiscordans* (A2 from Navarra) was recovered within *Coccomyxa* (Fig. 8). The common presence of epibionts, including some that resembled *Coccomyxa*, was evident in TEM images of the lichen thallus exterior and particularly in the cultured isolates.

Discussion

The pyrenoid type observed within thalli of *Byssoloma subdiscordans*, *Fellhanera bouteillei*, *Bacidina apiahica* and *Tapellaria epiphylla* corresponds in structure to that observed in the alga *Chloroidium saccharophilum* (Ikeda & Takeda 1995; González

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Table 1. GenBank Accession numbers corresponding to the *rbcL* and nrSSU sequences obtained during this study. Locality: N = Navarra; T = Tenerife. Culture: Y = Yes; N = No.

Locality	Lab Code	Photobiont	Mycobiont	Culture	*rbcL* Accession number	nrSSU Accession number
T	E134	*Chloroidium* sp.	*Byssoloma kakouettae*	Y	OR751262	OR750715
T	A31	*Symbiochloris* sp.	*Byssoloma leucoblepharum*	N		OR750707
T	A32	*Symbiochloris* sp.	*Byssoloma leucoblepharum*	N		OR750708
N	Arb1	*Chloroidium* sp.	*Byssoloma subdiscordans*	N		OR750709
T	A25	*Chloroidium* sp.	*Byssoloma subdiscordans*	N	OR751268	
T	A2	*Coccomyxa* sp.	*Byssoloma subdiscordans*	N		OR750705
T	A2	*Chloroidium* sp.	*Byssoloma subdiscordans*	N		OR750706
T	E135	*Chloroidium* sp.	*Byssoloma subdiscordans*	Y	OR751274	OR750716
N	Arb25	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751267	
N	Arb43	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751270	
N	Arb44	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751266	
N	Arb46	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751269	
N	Arb49	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751273	
N	Arb50	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751272	
N	Arb21	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751271	OR750711
N	Arb22	*Chloroidium* sp.	*Fellhanera bouteillei*	N	OR751265	OR750712
N	Arb42	*Trebouxia* sp.	*Fellhanera bouteillei*	N		OR750713
N	Arb6	*Chloroidium* sp.	*Fellhanera bouteillei*	N		OR750710
N	E136	*Chloroidium* sp.	*Fellhanera bouteillei*	Y	OR751264	OR750717
N	FA2	*Chloroidium* sp.	*Fellhanera bouteillei*	N		OR750719
T	A16	*Chloroidium* sp.	*Gyalectidium minus*	N		OR750714
T	E137	*Chloroidium* sp.	*Tapellaria epiphylla*	Y	OR751263	OR750718

et al. 2013), the type species of that genus (Darienko *et al.* 2010). For *C. lichenum*, we found no images or descriptions of pyrenoid ultrastructure in the literature. The presence of *Chloroidium* phycobionts associated with *B. subdiscordans* and *F. bouteillei* in both foliicolous communities studied here was supported by nrSSU and/or *rbcL* sequence data (Figs 7 & 8). Although attempts to obtain sequences from *Bacidina apiahica* and *T. epiphylla* thalli were unsuccessful, TEM observation of within-thallus phycobionts showing the same kind of pyrenoid strongly suggests that these lichens also partner with *Chloroidium*. This appears to be the first published report of the algal genus *Chloroidium* occurring as symbiont in foliicolous communities. Elsewhere, species of *Chloroidium* have been reported as phycobionts of diverse lichen-forming fungi, such as certain species of crustose *Trapelia* (Tschermak-Woess 1988; Beck 2002; Peršoh *et al.* 2004) and *Verrucaria* (Thüs *et al.* 2011; Voytsekhovitch & Beck 2016), squamulose *Psora* (Ruprecht *et al.* 2014), foliose *Sticta* (Lindgren *et al.* 2020), and fruticose *Stereocaulon* (Vančurová *et al.* 2018), as well as occurring as non-lichenized members of aquatic and terrestrial communities (Darienko *et al.* 2010; González *et al.* 2013; Metz *et al.* 2019; Li *et al.* 2021). The presence of *Chloroidium* as symbiont in the specialized phyllosphere community further highlights the considerable ecological range of this genus.

Heveochlorella, the sole phycobiont found associated with *Gomphillaceae* and *Pilocarpaceae* in the Floridian foliicolous community studied previously (Sanders *et al.* 2016), also appeared to be a symbiont in the Spanish foliicolous communities studied here. Its characteristic pyrenoid type (Zhang *et al.* 2008; Ma *et al.* 2013; Sanders *et al.* 2016) was observed in thalli of *G. setiferum* (Navarra) and *G. minus* (Tenerife), predominant elements in their respective leaf communities. However, molecular sequence data were somewhat less supportive in the case of the former, and contradictory in the case of the latter. For *G. setiferum*, all obtained sequences showed a number of double peaks; in BLAST searches, they returned *Heveochlorella*/*Jaagichlorella* sequences, albeit with low percentage identity (data not shown). Of more problematic interpretation were the sequences obtained from *G. minus*, the one clean sequence pointing to *Chloroidium lichenum*, which is clearly at odds with the pyrenoid type observed. Based on the TEM observations, we are fairly confident that *Heveochlorella* was the phycobiont present in at least the thallus we sectioned, but we cannot rule out the possibility that *Chloroidium* was phycobiont in the thalli we sequenced, particularly since it occurs as phycobiont in other lichens of the same community. We are considerably more doubtful that the *Coccomyxa* sequence obtained from *Byssoloma subdiscordans* and the *Trebouxia* sequence obtained from *Fellhanera bouteillei* thalli represent phycobionts of the thalli in question. *Coccomyxa* lacks pyrenoids and has a rather distinctive chloroplast ultrastructure that was not seen among phycobionts within any foliicolous thalli examined in the present or previous studies. And while the

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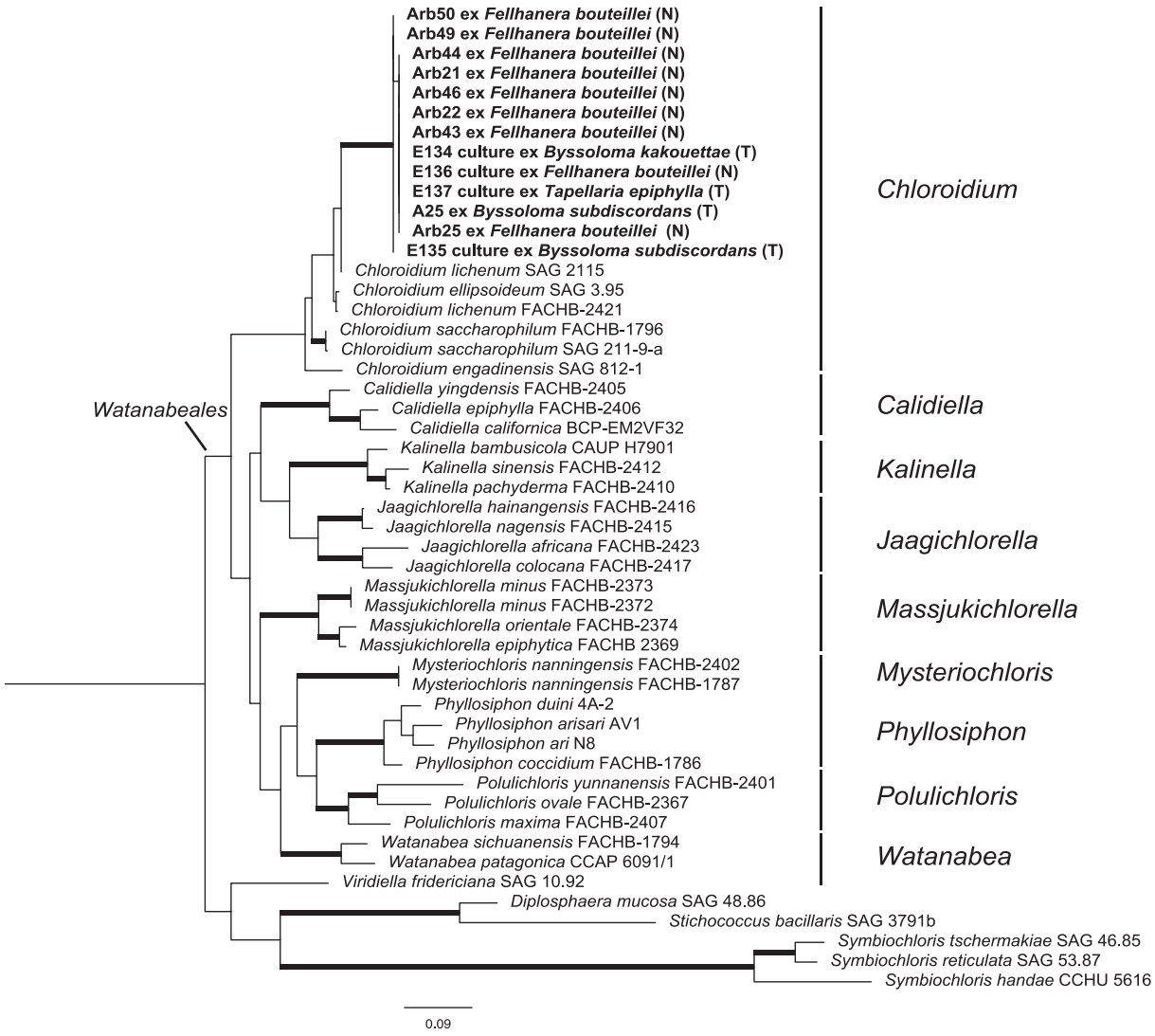

Figure 7. Most-likely tree inferred by maximum likelihood (ML) analysis of the *rbcL* region, showing the relationships of photobionts of the studied foliicolous species with members of the *Watanabeales*. Thick branches indicate nodes with bootstrap values ≥ 70%. Locality: (N) = Navarra; (T) = Tenerife.

pyrenoids present in *Chloroidium* and *Heveochlorella* each have counterparts within the genus *Trebouxia*, the typical autospore packet of 8–16 cells that is characteristic of *Trebouxia* was not observed in any foliicolous lichen thallus. Epibiontic algae may well have been responsible for the sequence ambiguities, but it is also plausible that more than one phycobiont genus might associate with a particular mycobiont in these communities. The minute size of foliicolous lichens, particularly *Gyalectidium* species, introduces several difficulties in obtaining accurate sequences from their phycobionts. Epibionts may be abundant in these communities, particularly where older leaves and thalli are concerned, but cannot be cleaned from the surfaces of such tiny thalli. Furthermore, to obtain sufficient DNA, several neighbouring thalli are needed, while yet others must be processed for TEM observation, introducing the possibility that DNA sequences, even when they accurately reflect the phycobiont of the thallus sampled, might not always correspond to the one present in the separate sample from which TEM images are obtained. These same uncertainties also apply to the algal cultures established in this study since the thallus areolae or fragments used as inoculum were too small to clean or surface-sterilize. It is furthermore possible that individual thalli contain more than one photobiont species, as has been reported in a number of lichens colonizing other substrata (e.g. Piercey-Normore 2006; Muggia *et al.* 2014; Park *et al.* 2015; Dal Grande *et al.* 2018; Osyczka *et al.* 2021). The ontogeny of the foliicolous thallus can offer many opportunities for multiple photobionts to be incorporated into a single thallus (Sanders 2014), although we have not observed algal cells with clearly different pyrenoid types occurring as phycobiont within the same thallus.

The distinct pyrenoid types characteristic of *Chloroidium* and *Heveochlorella* phycobionts are compared and contrasted in Table 2. It should be noted that other taxa of *Chloroidium* are said to lack pyrenoids (Darienko *et al.* 2010), and it is also conceivable that species not yet examined with TEM might be found to have a type of pyrenoid different from those reported so far. At least five or six different pyrenoid types are known in the single phycobiont genus *Trebouxia* s. str. (Friedl 1989; Bordenave *et al.* 2022). Remarkably, the two pyrenoid morphologies observed here in the *Watanabeales* correspond rather closely to two of the pyrenoid types known from *Trebouxia* (*Trebouxiales*): the pyrenoid of *Heveochlorella* is very similar to the [*Trebouxia*] *impressa*-type, while that of the *Chloroidium*

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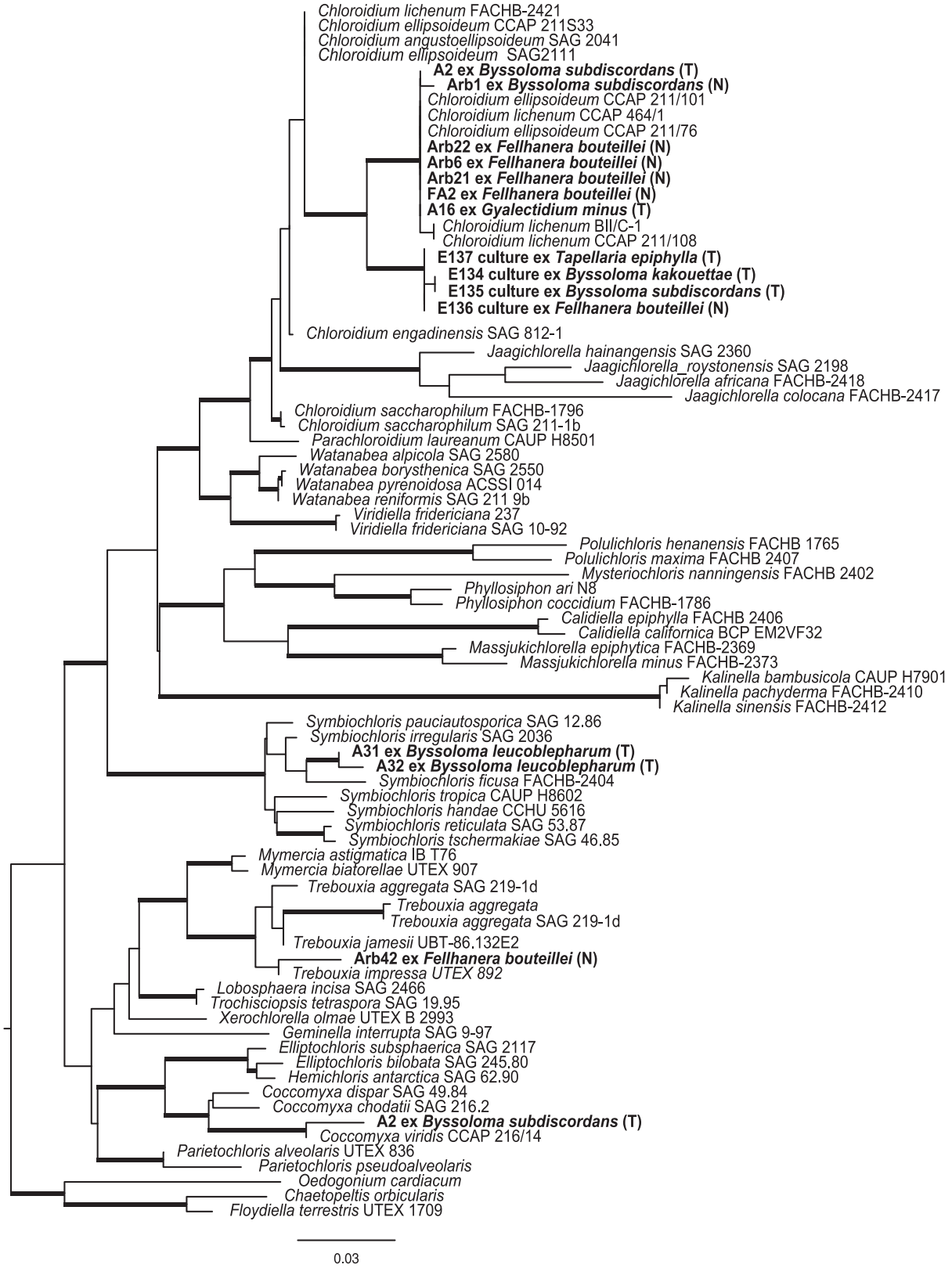

Figure 8. Most-likely tree inferred by maximum likelihood (ML) analysis of the nrSSU region, showing the relationships of photobionts of the studied foliicolous species with members of the *Trebouxiophyceae*. Thick branches indicate nodes with phylogenetic support in both analyses (bootstrap values ≥ 70% and posterior probability ≥ 0.95). Locality: (N) = Navarra; (T) = Tenerife.

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Table 2. A comparison of pyrenoid characteristics of *Heveochlorella* and *Chloroidium* strains.

Pyrenoid character	*Heveochlorella* sp.	*Chloroidium* sp.
Origin of penetrating membrane structures	continuous with outer membranes of thylakoid stack	continuous with single thylakoids
Morphology	centripetal tubules	sinuous plates
Width	equal to stack of several thylakoids	equal to one thylakoid
Position of pyrenoglobuli	lining the tubules	at periphery of pyrenoid

specimens is similar to the *corticola*-type, as discussed above. The *Trebouxiales* and *Watanabeales* are not closely related within *Trebouxiophyceae*. There is evidence linking pyrenoids to mechanisms of concentrating CO_2 in the vicinity of Rubisco (Palmqvist *et al.* 1997). Penetrating membranes appear to lack O_2-generating photosystem II and are thought to play a role in CO_2 delivery (Meyer *et al.* 2017), but the functional significance of pyrenoid structural diversity is largely unknown. The remarkable convergence of pyrenoid types among distantly related genera suggests that substantial selection pressures act upon functional morphology, with a relatively limited range of structural solutions available.

In contrast with those of *Heveochlorella* and *Chloroidium*, the pyrenoids seen in the phycobiont of *Byssoloma leucoblepharum* were much less clearly delimited and lacked any specialized penetrating membranes (Fig. 5). A BLAST search with two clean nrSSU sequences from this lichen indicated *Symbiochloris*, an algal genus not known to have pyrenoids (Škaloud *et al.* 2016). It is conceivable that the poorly differentiated pyrenoids were overlooked previously, or not judged to be true pyrenoids, but the chloroplast morphology we observed in *B. leucoblepharum* phycobionts does not appear to show the reticulate structure characteristic of *Symbiochloris* and *Dictyochloropsis*. It is possible that surface epibionts were amplified, or that the thallus observed in TEM contained an alga different from those genetically sampled. Although *Symbiochloris* partners with a considerable diversity of lichen-forming fungi (Sanders & Masumoto 2021) and appears to occur abundantly in phyllosphere communities of tropical Asia (Zhu *et al.* 2018), it has not been reported as a foliicolous lichen symbiont. For the lichen *Byssoloma kakouettae*, while TEM images of a cultured isolate point to *Chloroidium* as phycobiont, the pyrenoid type observed within the thallus was diffuse and poorly differentiated. Thus, we were not able to adequately resolve the algal partner identities for *Byssoloma kakouettae* and *B. leucoblepharum* in the communities examined, nor confirm the status of *Symbiochloris* as a potential foliicolous lichen symbiont. Our results highlight both the strengths and limitations of using in-thallus TEM images of phycobionts to help determine their identities. Where distinctive pyrenoids characterize the strains involved, such as those of *Chloroidium* versus *Heveochlorella*, the TEM images can provide an important check against molecular sequence data that might actually correspond to an epibiont rather than the true thallus symbiont. This is particularly helpful with foliicolous lichens, whose thalli are generally too small to effectively clean of epibionts. In other cases, however, obvious differences in pyrenoid ultrastructure may not be apparent between candidate phycobionts of even quite different genera. Nonetheless, further TEM studies may provide additional details that help resolve some ambiguities. For example, while the pyrenoid observed in *Heveochlorella* largely corresponds to the *impressa*-type found in some *Trebouxia* species (Friedl 1989; Barreno *et al.* 2022), the ultrastructure of the chloroplast near the pyrenoid periphery looks quite different in *Trebouxia* species with this type of pyrenoid. Dilation of thylakoids with electron-transparent lumina is evident in the *Trebouxia* chloroplast well before the membranes enter the pyrenoid as tubules, and they do not appear to arise from the outermost membranes of a multi-thylakoid stack (Barreno *et al.* 2022; Bordenave *et al.* 2022) as they do in *Heveochlorella* (Fig. 4B; Sanders *et al.* 2016, fig. 3D & F).

While the presence of surrounding starch plates often figures as part of pyrenoid type descriptions, we found this to be a variable character in the present study. Surrounding starch plates were consistently present in the *Heveochlorella* phycobiont pyrenoids observed from Florida, even in cultured isolates (Sanders *et al.* 2016), but were absent from phycobionts of the two species of *Gyalectidium* observed in the two habitats of the present study (Fig. 4). Starch plates were clearly visible surrounding the pyrenoids of *Chloroidium* associated with *Byssoloma subdiscordans* and *Fellhanera bouteillei* from Navarra (Fig. 1) but were absent in the same two lichen species from Tenerife, as well as *Bacidina apiahica* from that locality (Fig. 2). TEM micrographs of *Chloroidium* sampled from free-living collections (Ikeda & Takeda 1995; González *et al.* 2013) did not show visible starch accumulations surrounding the pyrenoid. It seems likely that local or seasonal variability in resource accumulation and use might contribute to the presence or absence of starch plates observed in a sample at a given time. Within the same culture of *Chloroidium* isolated from *Byssoloma kakouettae*, some cells showed relatively abundant pyrenoglobuli and only limited starch deposits surrounding the pyrenoid, while others showed the opposite pattern (Fig. 6C & D). While the degree of usage and storage of these carbohydrate and lipid resources appears to vary from cell to cell, their location appears to be characteristic of the pyrenoid type, and in the case of the pyrenoglobuli, that location is distinctly different in the *Chloroidium* and *Heveochlorella* pyrenoids observed (Table 2).

Relatively few sequences are currently available for foliicolous lichen phycobionts, and most are from outside the principal centre of diversity of these communities in the Neo- and Paleotropics. Among the challenges involved are the apparent instability of the genetic material, and the difficulties in obtaining clean sequences from very tiny thalli growing among diverse microalgae of the phyllosphere. Nonetheless, further efforts are likely to prove rewarding if these technical difficulties can be resolved. The adaptations of foliicolous lichens to the unique conditions of the ephemeral leaf substratum, the diversity of their sexual and asexual means of reproduction, and their symbiotic as well as aposymbiotic dispersal of propagules pose many interesting questions worthy of further exploration, particularly in regard to symbiont selection and the generational continuity of genetic partnerships.

Acknowledgements. Ultrathin sections were prepared by Beatriz Martín Jouve (Centro Nacional de Biotecnología, CSIC, Madrid). The collection of foliicolous lichens at Anaga was facilitated by the Cabildo de Tenerife, Área de Sostenibilidad, Medio Ambiente y Seguridad (permit # O00006501s1900019469). WBS is grateful to Dr Javier Etayo and his wife Eva for their generous hospitality during a collecting trip to Navarra, and to Dr Israel Pérez Vargas for kindly providing laboratory facilities during a similar visit to Tenerife. Funding

https://doi.org/10.1017/S0024282924000069 Published online by Cambridge University Press

was provided by an FGCU Scholarship-Research Venture Capital Award. The manuscript benefited from peer review by Dr Pavel Škaloud and two anonymous referees.

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Competing Interests. The authors declare none.

Supplementary Material. The supplementary material for this article can be found at https://doi.org/10.1017/S0024282924000069.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Barreno E, Muggia L, Chiva S, Molina A, Bordenave C, García-Brejo F and Moya P (2022) *Trebouxia lynnae* sp. nov. (former *Trebouxia* sp. TR9): biology and biogeography of an epitome lichen symbiotic microalga. *Biology* **11**, 1196.

Beck A (2002) *Selektivität der symbionten schwermetalltoleranter Flechten*. Ph.D. thesis, Ludwig Maximilian University of Munich.

Bordenave CD, Muggia L, Chiva S, Leavitt SD, Carrasco P and Barreno E (2022) Chloroplast morphology and pyrenoid ultrastructural analyses reappraise the diversity of the lichen phycobiont genus *Trebouxia* (*Chlorophyta*). *Algal Research* **61**, 102561.

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**, 540–552.

Dal Grande F, Beck A, Cornejo C, Singh G, Cheenacharoen S, Nelsen MP and Scheidegger C (2014) Molecular phylogeny and symbiotic selectivity of the green algal genus *Dictyochloropsis* s. l. (*Trebouxiophyceae*): a polyphyletic and widespread group forming phycobiont-mediated guilds in the lichen family *Lobariaceae*. *New Phytologist* **202**, 455–470.

Dal Grande F, Rolshausen G, Divakar PK, Crespo A, Otte J, Schleuning M and Schmitt I (2018) Environment and host identity structure communities of green algal symbionts in lichens. *New Phytologist* **217**, 277–289.

Darienko T and Pröschold T (2019) The genus *Jaagichlorella* Reisigl (*Trebouxiophyceae*, *Chlorophyta*) and its close relatives: an evolutionary puzzle. *Phytotaxa* **388**, 47–68.

Darienko T, Gustavs L, Mudimu O, Menendez CR, Schumann R, Karsten U, Friedl T and Pröschold T (2010) *Chloroidium*, a common terrestrial coccoid green alga previously assigned to *Chlorella* (*Trebouxiophyceae*, *Chlorophyta*). *European Journal of Phycology* **45**, 79–95.

Darienko T, Lukešova A and Pröschold T (2018) The polyphasic approach revealed new species of *Chloroidium* (*Trebouxiophyceae*, *Chlorophyta*). *Phytotaxa* **372**, 51–66.

de los Ríos A and Ascaso C (2002) Preparative techniques for transmission electron microscopy and confocal laser scanning microscopy of lichens. In Kranner IC, Beckett RP and Varma AK (eds), *Protocols in Lichenology*. Berlin and Heidelberg: Springer-Verlag, pp. 87–117.

Friedl T (1989) Comparative ultrastructure of pyrenoids in *Trebouxia* (*Microthamniales*, *Chlorophyta*). *Plant Systematics and Evolution* **164**, 145–159.

González MA, Pröschold T, Palacios Y, Aguayo P, Ibostroza I and Gómez PI (2013) Taxonomic identification and lipid production of two Chilean *Chlorella*-like strains isolated from a marine and an estuarine coastal environment. *AoB Plants* **5**, plt020.

Ikeda T and Takeda H (1995) Species-specific differences of pyrenoids in *Chlorella* (*Chlorophyta*). *Journal of Phycology* **31**, 813–818.

Katoh K, Misawa K, Kuma K I and Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**, 3059–3066.

Li S, Tan H, Liu B, Zhu H, Hu Z and Liu G (2021) *Watanabeales* ord. nov. and twelve novel species of *Trebouxiophyceae* (*Chlorophyta*). *Journal of Phycology* **57**, 1167–1186.

Lindgren H, Moncada B, Lücking R, Magain N, Simon A, Goffinet B, Sérusiaux E, Nelsen MP, Mercado-Díaz JA, Widhelm TJ, *et al.* (2020) Cophylogenetic patterns in algal symbionts correlate with repeated symbiont switches during diversification and geographic expansion of lichen-forming fungi in the genus *Sticta* (*Ascomycota*, *Peltigeraceae*). *Molecular Phylogenetics and Evolution* **150**, 106860.

Lücking R (2001) Lichens on leaves in tropical rainforests: life in a permanently ephemerous environment. *Dissertationes Botanicae* **346**, 41–77.

Lücking R (2008) Foliicolous lichenized fungi. *Flora Neotropica Monograph* **103**, 1–866.

Ma S, Huss VAR, Tan D, Sun X, Chun J, Xie Y and Zhang J (2013) A novel species in the genus *Heveochlorella* (*Trebouxiophyceae*, *Chlorophyta*) witnesses the evolution from an epiphytic into an endophytic lifestyle in tree-dwelling green algae. *European Journal of Phycology* **48**, 200–209.

Manhart JR (1994) Phylogenetic analysis of green plant *rbcL* sequences. *Molecular Phylogenetics and Evolution* **3**, 114–127.

Metz S, Singer D, Domaizon I, Unrein F and Lara E (2019) Global distribution of *Trebouxiophyceae* diversity explored by high-throughput sequencing and phylogenetic approaches. *Environmental Microbiology* **21**, 3885–3895.

Meyer M, Whittaker C and Griffiths H (2017) The algal pyrenoid: key unanswered questions. *Journal of Experimental Botany* **68**, 3739–3749.

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

Neustupa J, Němcová Y, Eliáš M and Škaloud P (2009) *Kalinella bambusicola* gen. et sp. nov. (*Trebouxiophyceae, Chlorophyta*), a novel coccoid *Chlorella*-like subaerial alga from Southeast Asia. *Phycological Research* **57**, 159–169.

Osyczka P, Lenart-Boroń A, Boroń P and Rola K (2021) Lichen-forming fungi in postindustrial habitats involve alternative photobionts. *Mycologia* **113**, 43–55.

Palmqvist K, de los Ríos A, Ascaso C and Samuelsson G (1997) Photosynthetic carbon acquisition in the lichen phycobionts *Coccomyxa* and *Trebouxia* (*Chlorophyta*). *Physiologia Plantarum* **101**, 67–76.

Park CH, Kim KM, Elvebakk A, Kim O-S, Jeong G and Hong SG (2015) Algal and fungal diversity in Antarctic lichens. *Journal of Eukaryotic Microbiology* **62**, 196–205.

Peršoh D, Beck A and Rambold G (2004) The distribution of ascus types and photobiontal selection in *Lecanoromycetes* (*Ascomycota*) against the background of a revised SSU nrDNA phylogeny. *Mycological Progress* **3**, 103–121.

Piercey-Normore MD (2006) The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. *New Phytologist* **169**, 331–344.

Ruprecht U, Brunauer G and Türk R (2014) High phycobiont diversity in the common European soil crust lichen *Psora decipiens*. *Biodiversity and Conservation* **23**, 1771–1785.

Sanders WB (2014) Complete life cycle of the lichen fungus *Calopadia puiggarii* (*Pilocarpaceae*, *Ascomycetes*) documented in situ: propagule dispersal, establishment of symbiosis, thallus development, and formation of sexual and asexual reproductive structures. *American Journal of Botany* **101**, 1836–1848.

Sanders WB and Masumoto H (2021) Lichen algae: the photosynthetic partners in lichen symbioses. *Lichenologist* **53**, 347–393.

Sanders WB, Pérez-Ortega S, Nelsen MP, Lücking R and de los Ríos A (2016) *Heveochlorella* (*Trebouxiophyceae*): a little-known genus of unicellular green algae outside the *Trebouxiales* emerges unexpectedly as a major clade of lichen phycobionts in foliicolous communities. *Journal of Phycology* **52**, 840–853.

Santesson R (1952) Foliicolous lichens I. *Symbolae Botanicae Upsaliensis* **12**, 1–590.

Škaloud P, Friedl T, Hallmann C, Beck A and Dal Grande F (2016) Taxonomic revision and species delimitation of coccoid green algae currently assigned to the genus *Dictyochloropsis* (*Trebouxiophyceae*, *Chlorophyta*). *Journal of Phycology* **52**, 599–617.

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313.

Thüs H, Muggia L, Pérez-Ortega S, Favero-Longo SE, Joneson S, O'Brien H, Nelsen MP, Duque-Thüs R, Grube M, Friedl T, *et al.* (2011)

Revisiting phycobiont diversity in the lichen family *Verrucariaceae* (*Ascomycota*). *European Journal of Phycology* **46**, 399–415.

Tschermak-Woess E (1988) The algal partner. In Galun M (ed.), *CRC Handbook of Lichenology*. Bca Raton, Florida: CRC Press, pp. 39–92.

van den Boom PPG (2020) Foliicolous lichens and their lichenicolous fungi in Macaronesia and Atlantic Europe. *Bibliotheca Lichenologica* **111**, 1–197.

Vančurová L, Muggia L, Peksa O, Řídká T and Škaloud P (2018) The complexity of symbiotic interactions influences the ecological amplitude of the host: a case study in *Stereocaulon* (lichenized *Ascomycota*). *Molecular Ecology* **27**, 3016–3033.

Verbruggen H, Ashworth M, LoDuca ST, Vlaeminck C, Cocquyt E, Sauvage T, Zechman FW, Littler DS, Littler MM, Leliaert F, *et al.* (2009) A multilocus time-calibrated phylogeny of the siphonous green algae. *Molecular Phylogenetics and Evolution* **50**, 642–653.

Voytsekhovich A and Beck A (2016) Lichen phycobionts of the rocky outcrops of Karadag massif (Crimean Peninsula). *Symbiosis* **68**, 9–24.

Zhang J, Huss VAR, Sun X, Chang K and Pang D (2008) Morphology and phylogenetic position of a trebouxiophycean green alga (*Chlorophyta*) growing on the rubber tree, *Hevea brasiliensis*, with the description of a new genus and species. *European Journal of Phycology* **43**, 185–193.

Zhu H, Li S, Hu Z and Liu G (2018) Molecular characterization of eukaryotic algal communities in the tropical phyllosphere based on real-time sequencing of the 18S rDNA gene. *BMC Plant Biology* **18**, 365.

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