Chloroidium phycobionts (Watanabeales, Trebouxiiophyceae) 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, Trebouxiiophyceae) 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 because of 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

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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 trebouxiiophycean 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...
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
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 kakouetae (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 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.
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 H₂O, 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 H₂O, 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 MgCl₂ 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 mm²), 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

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.
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 cardiaum, Chaetopeltis orbicularis and Floydia 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?task.cgi?task_type=gblocks) applying all the options available for the least stringent selection. The alignments were analyzed using maximum likelihood and Bayesian inference methods, and the best models were selected using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC).
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. 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 (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 bouteili from both Navarra and Tenerife (Figs 1 & 2), as well as in those of Bacidina apiatihca (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).

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sequences, both from thalli and cultures from Tenerife and Navarra, formed a well-supported clade within the genus *Chloroidium*, sister to *C. angustoellipsoides* 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. ellipsoides*, *C. lichenoides*, and *C. angustoellipsoides* 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 *Trebovia* 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 apihica*, and *Tapellaria ephylla* corresponds in structure to that observed in the alga *Chloroidium saccharophilum* (Ikeda & Takeda 1995; González...
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 apiaphica 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 (Thús et al. 2011; Voytekshovitch & 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), that, as referred to in previous studies, the pyrenoids and has a rather distinctive chloroplast ultrastructure observed. Based on the TEM observations, we are fairly confident that we observed. Based on the TEM observations, we are fairly confident that Heveochlorella was the phycobiont present in at least 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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<td>Y</td>
<td>OR751264</td>
<td>OR750717</td>
</tr>
<tr>
<td>N</td>
<td>FA2</td>
<td>Chloroidium sp.</td>
<td>Fellhanera bouteillei</td>
<td>N</td>
<td>OR750719</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>A16</td>
<td>Chloroidium sp.</td>
<td>Gyalectidium minus</td>
<td>N</td>
<td>OR750714</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>E137</td>
<td>Chloroidium sp.</td>
<td>Tapellaria epiphylla</td>
<td>Y</td>
<td>OR751263</td>
<td>OR750718</td>
</tr>
</tbody>
</table>

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.
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. Piercy-Normore 2006; Muggia et al. 2014; Park et al. 2015; Dal Grande et al. 2018; Oszczka 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*
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 \( \geq 70\%) and posterior probability \( \geq 0.95\)). Locality: (N) = Navarra; (T) = Tenerife.
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 CO₂ in the vicinity of Rubisco (Palmqvist et al. 1997). Penetrating membranes appear to lack O₂-generating photosystem II and are thought to play a role in CO₂ 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 Chlororidium, the pyrenoids seen in the photobiont 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 (Skaloud 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 photobionts 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 & Masamoto 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 kakouettaceae, while TEM images of a cultured isolate point to Chlororidium as photobiont, 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 kakouettaceae 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 photobionts to help determine their identities. Where distinctive pyrenoids characterize the strains involved, such as those of Chlororidium 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 photobionts 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; Barredo 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 photobionts 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 Chlororidium associated with Byssoloma subsidicordians and Fellhanera bouteillei from Navarra (Fig. 1) but were absent in the same two lichen species from Tenerife, as well as Bacidina apiaihica from that locality (Fig. 2). TEM micrographs of Chlororidium 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 Chlororidium isolated from Byssoloma kakouettaceae, 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 Chlororidium and Heveochlorella pyrenoids observed (Table 2).

Relatively few sequences are currently available for foliicolous lichen photobionts, 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.

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Table 2. A comparison of pyrenoid characteristics of Heveochlorella and Chlororidium strains.

<table>
<thead>
<tr>
<th>Pyrenoid character</th>
<th>Heveochlorella sp.</th>
<th>Chlororidium sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of penetrating membrane structures</td>
<td>continuous with outer membranes of thylakoid stack</td>
<td>continuous with single thylakoids</td>
</tr>
<tr>
<td>Morphology</td>
<td>centripetal tubules</td>
<td>sinuous plates</td>
</tr>
<tr>
<td>Width</td>
<td>equal to stack of several thylakoids</td>
<td>equal to one thylakoid</td>
</tr>
<tr>
<td>Position of pyrenoglobuli</td>
<td>lining the tubules</td>
<td>at periphery of pyrenoid</td>
</tr>
</tbody>
</table>

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**Competing Interests.** The authors declare none.

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