A multi-tool approach to assess microalgal diversity in lichens: isolation, Sanger sequencing, HTS and ultrastructural correlations

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ORIGINAL ARTICLES

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Running Head: Algal diversity by a multi-tool approach

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ABSTRACT

Lichen thalli represent the most relevant examples of fungal–algal interactions. Studies that describe phycobiont diversity within entire thalli are based mainly on Sanger sequencing. In some lichen species, this technique could underestimate the intrathalline coexistence of multiple microalgae. In this study, different multi-tool approaches were applied to two lichen taxa, *Circinaria hispida* and *Flavoparmelia soredians*, to detect algal coexistence. Here, we combined Sanger sequencing, a specific polymerase chain reaction (PCR) primer, 454-pyrosequencing, phycobiont isolation and ultrastructural characterisation. Furthermore, we compared pyrenoid ultrastructural features of lichenised phycobionts with microalgae isolated in culture. An improved methodology was used to isolate and propagate phycobionts which, in combination with fast genetic identification, resulted in a considerable reduction in time and cost to complete the process. This isolation method, coupled with a specific PCR primer, allowed for the detection of coexisting algae in *C. hispida* (four *Trebouxia* lineages). 454-pyrosequencing detected only a fraction of such diversity, while Sanger sequencing identified only the primary phycobiont. Ultrastructural features of the isolated algae were observed by transmission electron microscopy; the maintenance of the pyrenoid characteristics suggested the existence of different *Trebouxia* lineages. In *F. soredians*, a single *Trebouxia* lineage was identified using all these approaches.

In cases of lichens with algal coexistence, a combination of different molecular and ultrastructural approaches may be required to reveal the underlying algal diversity within a single thallus. The approaches proposed in this study provide information about the relationship between molecular and ultrastructural data, and they represent an improvement in

the delimitation of taxonomical features which is needed to recognise intrathalline *Trebouxia* diversity.

KEY WORDS

coexistence; propagation; 454-pyrosequencing; *Trebouxia.*

INTRODUCTION

Lichens are classical symbiotic associations (holobiont) that result in a complex organism with a specific biological organisation (Chapman & Margulis 1998). Such associations involve at least two very different organisms, a heterotrophic fungus (mycobiont) and a photosynthetic (photobiont). Photobionts can be cyanobacteria (cyanobionts) or green microalgae (phycobionts). The presence of microalgal communities, lichen-associated fungi, bacteria and yeast highlight the intrathalline complexity of lichen thalli (Arnold *et al*. 2009; U´Ren *et al*. 2010, 2012; Park *et al*. 2015; Aschenbrenner *et al*. 2016; Muggia *et al.* 2016a; Spribille *et al*. 2016; Moya *et al*. 2017).

Lichen literature has reported the occurrence of different phycobiont lineages inside the thalli in several lichen species (Blaha *et al*. 2006; Ohmura *et al*. 2006; Piercey-Normore 2006; Muggia *et al*. 2010, 2014; Schmull *et al*. 2011; Molins *et al*. 2013; Leavitt *et al*. 2015). The lichen *Ramalina farinacea* has proven to be a suitable model to study the multiplicity of microalgae in lichen thalli due to the recurrent coexistence of *Trebouxia* sp. TR9 and *T. jamesii* in long-distance populations (del Campo *et al.* 2010; Casano *et al.* 2011). Moreover, a recent study using a deep sequencing technique revealed a much higher microalgal diversity

associated with a single lichen thallus of *Ramalina farinacea* (22 operational taxonomic units [OTUs]) (Moya *et al*. 2017).

In order to understand the functions of microalgal communities in lichen thalli, phycobiont isolation is a key step in the experimental design. The isolation and culture of symbiotic organisms offers an opportunity to understand the mechanisms underlying the selection of partners in symbiotic interactions (Yoshimura *et al*. 2002). Cultures of lichen microalgae involve time-consuming techniques necessary for the successful propagation of these symbionts (Rafat *et al*. 2015). However, phycobiont isolation is an essential procedure to study lichen physiology, perform morphologic characterisation and obtain pure material for molecular identification.

Studies dealing with phycobiont diversity in lichen thalli are primarily based on Sanger sequencing. However, this technique could underestimate phycobiont diversity in some lichen species (del Campo *et al*. 2010; Molins *et al*. 2013). There has been an increase in the use of deep sequencing techniques to analyse the diversity of microalgal communities (Bates *et al*. 2012; Park *et al*. 2015) because these techniques allow for the detection of a vast number of genotypes that would otherwise remain underestimated in conventional PCR amplifications (Stewart & Cavanaugh 2009).

To understand the hidden intrathalline phycobiont diversity and the role of each phycobiont type in a symbiotic relationship, multi-tool and multidisciplinary approaches are needed to obtain comparative results (Catalá *et al*. 2015). In particular, we focused on three specific aims: i) to upgrade a methodology to propagate phycobionts that, coupled to fast molecular identification, will significantly reduce the time needed to complete the process; ii) to evaluate the phycobiont diversity using Sanger sequencing, 454-pyrosequencing and

isolation; and iii) to compare the ultrastructural characterisation between lichenised phycobionts and microalgae isolated in culture, and to correlate these ultrastructural traits using molecular analyses. To achieve these objectives, we selected *Circinaria hispida* (Mereschk.) A. Nordin, S. Savić and Tibell and *Flavoparmelia soredians* (Nyl.) Hale, two lichen species with different biotypes that occur in different ecosystems.

MATERIALS AND METHODS

Lichen material

The *Circinaria hispida* specimen was collected from Zaorejas (Guadalajara, Spain) (40°46'02"N, 2°11'40"W, 1105 m), and the *Flavoparmelia soredians* specimen was collected on *Quercus suber* cork from Chóvar (Castellón, Spain) (39°51'13,68"N, 0°19'09.60"W). Samples were dried out for one day and then stored at -20ºC until their processing.

Experimental design

Two lichen thalli (one per species) were examined under a stereo microscope to exclude surface contamination. Samples were rehydrated with Milli-Q sterile water one day before being processed, and they were stored in a growth chamber at 21ºC under a 12 h/12 h light/dark cycle (lighting conditions: 15 μmol/m^2s). Lichen thalli were vortexed three times for 5 min at 2,000 rpm with Milli-Q sterile water. Fragments from different parts of each thallus were randomly excised and homogenised together, and each pool was labelled as sample A, B, C or D. The mycobiont and primary phycobiont were identified by Sanger sequencing from sample A. Isolation protocol and Sanger identification was performed for

sample B. Sample C was analysed by 454-pyrosequencing. Phycobionts in sample D were characterised by transmission electron microscopy (TEM) to visualise ultrastructure (Fig.1).

A: DNA extraction, amplification and sequencing

Total genomic DNA from sample A was isolated and purified using the DNeasy TM Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (Fig. 1 Sample A). The phycobiont locus encoding the nrITS DNA (internal transcribed spacer) was amplified using the primer pair nr-SSU-1780 (Piercey-Normore & DePriest 2001) and ITS4 (White *et al*. 1990). Fungal nrITS DNA was amplified using the primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). PCR reactions were performed in 50 µl using the EmeraldAmp GT PCR Master Mix (Takara, Shiga, Japan), which required the addition of the template DNA, specific primers and water.

The PCR program for amplification was comprised of an initial denaturation cycle at 94ºC for 2 min; followed by 30 cycles at 94ºC for 30 sec, 56ºC for 45 sec and 72ºC for 1 min; and finally an elongation cycle at 72ºC for 5 min. Amplifications were carried out using a 96-well SensoQuest labcycler (Progen Scientific Ltd., South Yorkshire, UK). The PCR products were visualised on 2% agarose gels and purified using the Gel Band Purification Kit (GE Healthcare Life Science, Buckinghamshire, England). The amplified PCR products were sequenced with an ABI 3100 Genetic analyser using the ABI BigDye TM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California).

B: Isolation and culture conditions, fast identification and propagation

Isolation procedure: Phycobionts were isolated from sample B using the micromethod described by Gasulla *et al*. (2010). Samples were homogenised with a mortar and pestle in an isotonic buffer (0.3 M sorbitol, 50 mM HEPES, pH 7.5) and filtered through muslin. Isolation was carried out by a gradient centrifugation method using Percoll® (Fig. 1, Sample B).

Culture conditions: The algal suspension was diluted with sterile water for both lichen species and 10 µl was spread using the streak method on sterile 1.5% agar Bold's Basal Media (BBM) petri dishes (Bold 1949; Bischoff & Bold 1963). The isolated algae were maintained under a photosynthetic photon flux density (PPFD) of 15 µmol/m^2s with a 12 h photoperiod at 21°C; colonies began to develop under these conditions after 25–30 days.

Fast phycobiont identification and propagation: To perform a fast microalgae identification, PCR was performed directly from the well-developed colonies that were selected under the stereo microscope without carrying out DNA extraction. Algal colonies were picked using a sterile toothpick and introduced directly into the PCR mixture. Subsequently, the colonies were propagated directly by introducing this toothpick into a liquid medium (BBM). To check the subculture, a second PCR was performed on day 15 of cultivation without DNA extraction. All of the PCRs were performed under the same conditions as the PCR for sample A. The strains of isolated phycobionts were retained in our culture collection at the University of Valencia.

AB: Specific primer design

From the cultured strains sequenced in sample B, we designed specific forward primers (T1–T4, corresponding to the four isolated lineages; see the Results section). The primers were based on nrITS DNA differences among lineages, which allowed the PCR to

discriminate between the different isolated lineages (Fig. 1). The sequences of the primers were as follows:

- T1: 5´ TCACATTAAGCAATCAATTCTGAAGGCAGATCTACT 3´;
- T2: 5´ CCACTTTTAAGCAATCAATTCTGAAGGCAGATTTACA 3´;
- T3: 5´ TACCAGTCGGACTCACCTTGCCTTTG 3´; and
- T4: 5´ ATCTATAGGCTGGCTATGCTGGCTGTAGT 3´.

Semi-nested PCR was performed using the DNA template extracted from the thallus in sample A. We first used nr-SSU-1780/ITS4, followed by a nested reaction with the newly developed specific internal primers (T1–T4) and the reverse primer ITS4 (following the PCR conditions described for sample A).

Phylogenetic analysis of sequences obtained by Sanger sequencing

Two multiple alignments were prepared using ClustalW (Thompson *et al*. 1997). They included i) the newly determined algal nrITS DNA from the lichen thalli and from the colonies (KU318574 to KU318627 for *F. soredians,* and KU318629 to KU318666 for *C. hispida*); ii) some sequences of *Trebouxia* spp. retrieved from the GenBank — AJ249572 and KJ754251 for *C. hispida* and in the case of *F. soredians* KR914022 to KR914025, EU717918, JQ004553, JQ004570, JQ004571, JQ004578 and FJ792802;iii) selected sequences described by Leavitt *et al*. (2015) with 98%–99% identity and 100% coverage; and iv) a selection of *Trebouxia* species available from the Culture Collection of Algae at Goettingen University (SAG), species from the Culture Collection of Algae at the University of Texas (UTEX) and *Trebouxia* sp. TR9 (FJ418565). Variable and parsimony-informative characters were analysed with MEGA v 5.0 (Tamura *et al*. 2011). The jModelTest v 2.1.4 (Posada *et al*. 2009) with the

Akaike Information Criterion (Akaike 1974) was used to select the most appropriate model of nucleotide substitution (TVM+I+G in both alignments).

The phylogenetic relationships were estimated using Bayesian inferences (BI) and maximum likelihood (ML) inferences. Two parallel Markov Chain Monte Carlo (B/MCMC) runs were carried out using MrBAYES v 3.1.2 (Huelsenbeck & Ronquist 2003; Ronquist *et al*. 2005); each run used six chains simultaneously, was initiated with a random tree and was carried out for 10 million generations. The trees were sampled every 100th generation for a total sample of 200,000 trees.

ML trees were built in PhyML v 3.1 (Guindon *et al*. 2010) with 1,000 bootstrap replicates. Only clades that received bootstrap support above 90% in ML analyses and by posterior probabilities ≥0.9 in BI analyses were considered. The phylogenetic trees were visualised in TREEVIEW (Page 1996) and MEGA v 5.0 (Tamura *et al*. 2011).

To circumscribe OTUs that represent candidate lineages, we used the Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al*. 2012). We followed the conditions for *Trebouxia* spp that were reported by Leavitt *et al*. (2015). The ABGD program employs a genetic distance-based approach to detect OTUs that represent candidate species.

C: 454-pyrosequencing analyses

The over-amplification of the primary phycobiont was circumvented by performing RT-PCR. Rather than using a fixed PCR cycle number for all samples, the appropriate PCR cycle number for the 454-pyrosequencing assay was set based on the cycle threshold (Ct) (Moya et al. 2017). A first RT-PCR (RT-PCR I) and a first PCR (PCR I) were performed using the genomic DNA from sample C as a template and nr-SSU-1780/5.8S 2R primers (5.8S 2R;

Moya *et al*. 2017) (Fig. 1, Sample C). The number of cycles of PCR I (24 cycles for *C. hispida* and 23 for *F. soredians*) were determined by the average Ct of the RT-PCR I. Then we performed a second RT-PCR (RT-PCR II) and a second PCR (PCR II) using 1 µL of the PCR I as a template and the fusion primers designed following the GS Junior System Guidelines for Amplicon Experimental Design (Roche, Branford, USA). The specific cycle number for the PCR II (10 cycles for *C. hispida* and 11 for *F. soredians*) was determined by the average Ct from the RT-PCR II.

The RT-PCRs (20 µl) contained 10 µl of SYBR premix ExTaq (Takara, Shiga, Japan), 0.8 µM of each primer, 0.4 µl of ROX reference, 1 µl of template DNA and sterile Milli-Q water to volume. Each run contained quadruplicate samples using the following thermal cycle conditions: 30 s at 95ºC, followed by 40 cycles of 5 s at 95°C and 30s at 60 °C. In this analysis, the ABI StepOnePlus (Applied Biosystems, Foster City, CA, USA) was used; Ct values were determined using the StepOne software v 2.1 package (Applied Biosystems) and were based on fluorescence data recorded during each RT-PCR run.

The PCRs (25 µl) contained 2.5 µl of buffer 10X, 0.4 µM of each primer, 0.2 mM of dNTPs, 0.6 u/µl of ExTaq (Takara, Shiga, Japan) and sterile Milli-Q water to volume. The PCR conditions were one cycle of 95°C for 2 min; X number of cycles (24-23 PCR I, 10-11 PCR II) of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min; and a final extension of 72°C for 5 min. PCRs were performed in triplicate to prepare the amplicon generation libraries. The three PCR products from each sample were pooled together.

The amplicons were double purified using the Agencourt AMPure XP Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA). After purification, the amplicons were visualised in a Bioanalyzer 2100 and quantified by fluorometry using a Qubit

dsDNA BR Assay Kit (Invitrogen Molecular Probes, Eugene, Oregon, USA).

Algal nrITS DNA sequences were determined using a GS Junior 454 system (Roche 454 Life Sciences, Branford, CT, USA) following the Roche Amplicon Lib-L protocol at the Genomics Core Facility at the University of Valencia (Spain). Reads were processed for *Trebouxia* as described in Moya *et al.* (2017) and clustered based on a 99% score coverage threshold (-S 99) and 90% length coverage threshold (-L 0.9) criteria. The consensus sequences of the OTUs were identified using the BLAST tool in the GenBank data base (Altschul *et al*. 1990).

Phylogenetic analysis of sequences obtained by 454-pyrosequencing

Two multiple alignments were prepared. They included: i) the consensus sequence OTUs obtained by 454-pyrosequencing analysis; ii) a representative sequence of each of the isolated lineages (T1–T4 and F1); iii) and a selection of *Trebouxia* species available from the SAG, a selection from the UTEX and *Trebouxia* sp. TR9. Both alignments were carried out by MAFFT v 7.0 (Katoh *et al*. 2002; Katoh & Toh 2008) using default parameters. Alignments were 181 base pairs (bp) (*C. hispida*) and 197 bp (*F. soredians*) in length for the nrITS DNA region. The best-fit substitution model for this region (SYM+G for *C. hispida* and SYM for *F. soredians*) was chosen using jModelTest v 2.0 (Darriba *et al*. 2012) and by applying the Akaike Information Criterion (Akaike 1974). ML analysis was implemented in RAxML v 8.1.11 (Stamatakis 2006). ML searches were implemented using the GTRGAMMA substitution model. Bootstrap support was calculated based on 1,000 replications (Stamatakis *et al*. 2008). BI phylogenetic analyses were carried out using MrBAYES v 3.2 (Ronquist *et al*. 2012). Settings included two parallel runs; each run used six chains, was initiated with a

random tree and was carried out over 20 million generations, and sampling after every 200th generation. We discarded the first 25% of data as burn-in. MAFFT, jModelTest, ML and BI analyses were implemented at the CIPRES Science Gateway v 3.3 webportal (Miller *et al*. 2010). Phylogenetic trees were visualised in FigTree v. 1.4.1 (Rambaut 2012).

D: Microscopic examinations

TEM examinations were performed on sample D and on selected pure cultures on the day 21 of cultivation (Peksa & Škaloud 2008) from *C. hispida* and *F. soredians* (Fig. 1, Sample D).

The cells 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 then post-fixed with 2% OsO_4 in 0.01 M PBS (pH 7.4) for 2 h at room temperature. Thereafter, they were washed three times in 0.01 M PBS (pH 7.4) for 15 min and then dehydrated at room temperature in a graded ethanol series, (50%, 70%, 95% and 100%) for no less than 20–30 min at each step (Molins *et al*. 2013; Moya *et al*. 2015). 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). Sections of resin (90 nm) were cut with a diamond knife (DIATOME Ultra 45°) using an ultramicrotome (Reichert Ultracut E), mounted on oval hole copper grids, coated with formvar and post-stained with 2% (w/v) aqueous uranyl acetate and 2% lead citrate. The post-staining step was completed using the SynapTek Grid Staining Kit (http://www.ems-diasum.com/microscopy/technical/datasheet/71175.aspx). The sections were observed with a JEOL JEM-1010 (80 kV) electron microscope that was equipped with a MegaView III digital

camera and AnalySIS image acquisition software. TEM examinations were made at the SCSIE Service of the University of Valencia.

RESULTS

Identification of mycobiont and primary phycobiont by Sanger sequencing

The identity of the *C. hispida* and *F. soredians* mycobiont was confirmed by BLAST analyses against the GenBank database. Significant matches of 100% identity and 99% coverage were obtained with other *C. hispida* (HQ171233) and *F. soredians* (AY586562). The identity of the primary phycobiont present in both thalli was confirmed by BLAST searches of the nrITS DNA sequences and included in the phylogenetic analyses (Fig. 2, 3). Significant matches of 100% identity and 99% coverage were obtained with *Trebouxia cretacea* (KT819919) and *T. gelatinosa* (KR914022), respectively.

Trebouxia diversity by isolation

A total of 37 nrITS DNA sequences for *C. hispida* and 53 for *F. soredians* were obtained by sequencing directly from colonies. The aligned algal 5.8S and internal transcribed spacers (ITS1, ITS2) were 539 and 533 bp long, respectively. *C. hispida* showed 37 variable characters; however, there was only one variable character for *F. soredians*.

Preliminary phylogenetic analysis, which included our new dataset and the 69 sequences representative for the *Trebouxia* OTUs described by Leavitt *et al*. (2015) (data not shown), allowed us to select and include the most related OTU sequences in our phylogenetic analysis — A01 (*T. cretacea*, KT819919), A10 (*T. vagua*, KT819945), A12, A14, A20 (in the case of *C. hispida*), I05 and I15 (for *F. soredians*). BI and ML phylogenetic hypotheses were

297 topologically congruent; only clades supported by bootstrap ≥ 90% and posterior probabilities
298 ≥ 0.9 have been highlighted (Fig. 2, 3). The topology for the *Trebouxia* phylogenetic
299 relationship is congruent with previous studies (Muggia *et al*. 2010, 2014; Leavitt *et al.* 2015;
300 Voytsekhovich & Beck 2015).

301 All *Trebouxia* sequences from *C. hispida* belong to the `A´- *arboricola* group, and
302 they formed four statistically well-supported lineages named in the phylogenetic tree as T1,
303 T2, T3 and T4 (Fig.2). Lineage T1 fitted with *T. vagua* (OTU A10), lineage T2 formed a well-
304 supported new clade related to *T. asymmetrica*, lineage T3 matched with OTU A12 and
305 lineage T4 fitted with *T. cretacea* (OTU A01) and the primary phycobiont from the thallus
306 (underlined in the phylogenetic tree). Lineages T1, T3 and T4 were previously reported in
307 diverse lichen species. Only lineages T1 and T4 appeared in the genus *Circinaria*, T1 in *C.*
308 *contorta* and T4 in *A. desertorum* s.a. (Voytsekhovich & Beck 2015). Lineage T2 does not
309 match with any previously described *Trebouxia* sp. (Supplementary Data Table S1).

310 All the sequences obtained for *F. soredians* belong to the `I´- *impressa* group. They
311 formed a statistically well-supported lineage within *T. gelatinosa* (FJ626730, OTU I05)
312 named F1 and within other sequences ascribed to *T. gelatinosa* that are available in the
313 GenBank from diverse lichens (Fig. 3). Both ABGD and phylogenetic analyses delimited the
314 same lineages — T1–T4 for *C. hispida* and F1 for *F. soredians*.

315

316 **Intrathalline detection of isolated *Trebouxia* by using specific primers**

317 We designed specific forward primers to amplify any of the four *Trebouxia* lineages
318 detected in *C. hispida* based on their nrITS DNA. T1 to T4 were successfully amplified in the
319 *C. hispida* thallus (as indicated by the "○" symbol in the phylogenetic tree in Fig. 2). In the

case of *F. soredians,* only one *Trebouxia* lineage (F1) was detected, therefore a specific primer design was not necessary.

Trebouxia diversity by 454-pyrosequencing

Sequencing of nrITS amplicons produced 1,645 sequence reads for *C. hispida* and 1,272 for *F. soredians*. Singleton reads (43 and 19, respectively) were filtered out. By clustering with a 99% similarity cut-off, two OTUs for *C. hispida* (OTU1C, OTU2C) and one for *F. soredians* (OTU1F) were recognised. Tree reconstruction was congruent in all utilised methodologies (Supplementary Data Fig. S1, S2). The OTU1C matched with lineage T4 (*T. vagua*) and the OTU2C matched with lineage T3 (OTU A12). In the case of *F. soredians*, OTU1F fitted with *T. gelatinosa*.

Ultrastructural characterisation of microalgae

Comparative analyses were performed, based on the ultrastructure of pyrenoids, which allowed us to distinguish and compare algae isolated in culture (see left column in Fig. 4A, 4C, 4E, 4G, 6A) and in the respective lineages found in the symbiotic state (see right column in Fig. 4B, 4D, 4F, 4H, 6B). Pyrenoid types were determined according to Friedl (1989). The phycobionts of each lineage showed key structural features in common (Supplementary Data Fig. S3–S7).

Cells of lineage T1, both in symbiotic and in culture (Fig. 4A, 4B, Supplementary Data Fig. S3), showed the same large single pyrenoid type (*gigantea/impressa* type) with abundant and large pyrenoglobuli (130 to 150 nm, although in culture they may reach 300 nm). The pyrenoid was located in the centre of a single star-shaped lobed chloroplast; the

disposition of the thylakoid membranes was dense, with stackings of four or more straight membranes.

Cells of lineage T2, both in symbiotic and in culture (Fig. 4C, 4D, Supplementary Data Fig. S4), contained a central pyrenoid *gigantea* type, with some tubules of electron-dense content alternating with tubules of non-electron-dense content. The chloroplast was irregularly undulating in its outline, with a dense arrangement of slightly sinuous thylakoid stacks.

Cells of lineage T3, both in symbiotic and in culture (Fig. 4E, 4F, Supplementary Data Fig. S5), presented a new type of pyrenoid formed by small pyrenoidal structures that were distributed in the periphery and the central zone of the chloroplast. The chloroplast was irregularly crenated in its outline.

Lineage T4, both in symbiotic and in culture (Fig. 4G, 4H, Supplementary Data Fig. S6), had a large single pyrenoid *gigantea* type within homogeneous pyrenoglobuli and abundant non-electron-dense tubules. The chloroplast had an outline that was either irregularly undulating or deeper lobated with a loose arrangement of slightly sinuous thylakoid stacks. As shown in fig. 5, we detected coexistence inside the thallus of at least two lineages (T3 and T4).

F. soredians lineage F1 (Fig. 6A, 6B and Supplementary Data Fig. S7), in symbiotic and in culture, showed a large single *gelatinosa* type pyrenoid with tubules traversing the matrix in a parallel arrangement. Numerous pyrenoglobuli were connected to these tubules. The algal showed a large, lobed, dense chloroplast. The thylakoid membranes were grouped in scattered but evident grana.

DISCUSSION

Lichens represent micro-ecosystems suitable for the study of microorganism diversity in symbiotic associations. Traditional techniques could be biasing the results obtained by not identifying the full microalgal diversity.

The identification of *Circinaria hispida* and *Flavoparmelia soredians* was confirmed (KU318628 and KU318573) by the DNA barcoding proposed by Schoch *et al*. (2012) Molecular identification of lichenised fungi should not be overlooked in microalgal studies in order to avoid uploading *Trebouxia* sp. sequences to GenBank without any corresponding genetic information about their fungal partnerships (Catalá *et al*. 2015; Moya *et al*. 2015).

Sanger results detected only the primary phycobiont (T4 and F1). A potential bias in previous analyses is caused by using eukaryotic-specific primers to amplify the nrITS DNA; such primers could only detect the primary microalga, while other phycobionts remained undetected. Electropherograms showing double peaks or polymorphic sequences have been reported in lichens, but these samples were usually removed from analysis (Muggia *et al*. 2014; Leavitt *et al*. 2015; Voytsekhovich & Beck 2015). In such cases, a cloning strategy should be performed to separate the different sequences obtained (Molins *et al*. 2013), but this strategy does not allow us to obtain the algal cultures needed for ecophysiological and biotechnological studies.

454-pyrosequencing results detected two OTUs in *C. hispida* — OTU1C and OTU2C (matched with *T. cretacea*/T4 and OTU A12/T3) — and only one OTU in *F. soredians* — OTU1F (fitted with *T. gelatinosa*). The selected ITS1 and 5.8S fragments represent a variable region used to identify microalgae, and this is widely applied to discriminate *Trebouxia* species; however, 454-pyrosequencing using a GS Junior 454 system generated reads with

intermediate lengths that limited the number of base pairs in the genetic marker that was amplified. To avoid the over-amplification of the primary phycobiont, and to be able to detect possible OTUs present at intermediate abundances inside the thallus, it is necessary to determine, by a previous RT-PCR, the appropriate PCR cycle number to be applied for the preparation of the products to be pyrosequenced. The pyrosequencing assay performed in this study was included on a plate that comprised a total of 82 molecular identifiers. Only 1,645 and 1,272 filtered reads were recovered for *C. hispida* and *F. soredians*, respectively. In both cases, probable sequence variants at middle and low frequency were not uncovered.

An isolation procedure detected four *Trebouxia* lineages in *C. hispida* (T1–T4) and only one in *F. soredians* (F1). The phycobiont isolation method, based on the Percoll® gradient (Calatayud *et al*. 2001; Gasulla *et al*. 2010), provided a simple, effective and fast method for isolating and growing algal strains (no bacteria, hyphal fragments or spores were observed). This isolation method can be successfully used not only for *Trebouxia* isolation, but also for other coccoid green microalgae (such as *Asterochloris*, *Myrmecia* or *Coccomyxa*) from a broad range of lichens with diverse life forms. The procedure described in this study is an improvement to these methods because it makes it easy to separate and identify the potential algal diversity in a short period of time (2 days instead of 4–6 weeks), and it makes the molecular identification of microalgae in cultures affordable, something which is essential for physiological experiments.

A specific PCR primers confirmed the presence of the isolated *Trebouxia* lineages in the original DNA template (Sample A). In foliose and vagrant lichens, such as *F. soredians* and *C. hispida,* respectively, the thallus parts selected as starting material may represent an important factor that influences the final results, and therefore this is a key parameter to be

412 considered in the experimental design. Taking into account the differential localisation of the
413 phycobionts along the lacinias suggested by García *et al*. (2014) in *Ramalina farinacea*, we
414 decided to mix several parts of the thallus as starting material (Samples A, B, C and D) for
415 comparative analyses. Surprisingly, phycobiont isolation allowed us to detect more *Trebouxia*
416 lineages in *C. hispida* than by using 454-pyrosequencing, but these results can be explained
417 by i) the previously mentioned differential localisation of the phycobionts inside the thallus,
418 and ii) the low-coverage sequencing. Park *et al*. (2015) detected only one to three OTUs
419 (except in *Cladonia borealis*) when analysing several Antarctic lichen species, probably also
420 due to the low number of sequences recovered.

421 The same lichen thallus was used to perform different approaches to the analysis of
422 intrathalline microalgal diversity (Sanger sequencing, specific primer PCRs, 454-
423 pyrosequencing and isolation). These comparative analyses showed that the isolated
424 *Trebouxia* were indeed hosted in the thalli, but their differential detection was primarily
425 dependent on the protocol used, suggesting that the method and the zone selected in the
426 thallus may limit the detection of further associated algae due to methodologic biases. Further
427 studies, including the use of specimens from different populations, are needed to corroborate
428 the coexistence and non-coexistence patterns found in the lichens selected for the present
429 study.

430 Besides molecular techniques, phycobiont intrathalline co-occurrence was validated in
431 different lichen species through ultrastructural examinations by TEM (Casano *et al*. 2011;
432 Molins *et al*. 2013; Catalá *et al*. 2015). The ability to correlate molecular and ultrastructural
433 data, both in symbiotic and axenically isolated states, represents an improvement in clarifying
434 and delimiting the taxonomic concepts in the genus *Trebouxia*. Although algae undergo a

variety of modifications as a result of lichenisation (Galun 1988; Friedl & Büdel 2008), ultrastructural traits of pyrenoids from cultured phycobionts have been traditionally used to characterise *Trebouxia* species (Friedl 1989), and some authors have highlighted the suitability of the pyrenoid structures for species delimitation by TEM (Ascaso & Galván 1976; Ascaso *et al*. 1986; Brown *et al*. 1987; Catalá *et al*. 2015). Over the last few years, the ascertainment that *Trebouxia* diversity was much higher than previously considered highlights the need for a revision to the classification proposed by Friedl (1989) such that all of this heterogeneity in *Trebouxia* is included. The combination of genetic markers together with ultrastructural techniques, both in culture and in the symbiotic state, should be standardised in lichen research to make taxonomic concepts more accurate and to delimit *Trebouxia* diversity (Muggia *et al*. 2016).

In this study, the pyrenoid structures and the pyrenoglobuli arrangement in the phycobionts that were observed in *C. hispida* and *F. soredians* (Fig.4–6, Supplementary Data Fig. S3–S7) remained homogeneous enough within lichen thalli and in culture to allow for the correlation of both states. Few modifications were observed, probably due to the lichenisation process (e.g., cell wall thickness and the amount of mitochondria and ribosomes). To avoid modifications related to the conditions of the culture, we suggest standardising to BBM media culture for physiological experiments and selecting samples on day 21 of cultivation in order to perform ultrastructural analyses, as proposed by Muggia *et al*. (2016).

In the case of *C. hispida,* four *Trebouxia* lineages (T1–T4) were detected both inside the thallus and in isolated cultures (Fig. 4, Supplementary Data Fig. S3). T1 showed a pyrenoid that corresponded to the *impressa/gigantea* type, and T3 presented a new, easily

457 distinguishable pyrenoid type. T2 and T4 demonstrated similar pyrenoid types, corresponding
458 to the *gigantea* type described by Friedl (1989). Pyrenoid similarities between these lineages
459 agreed with their phylogenetic closeness (Fig. 2). In this case, the stability of the chloroplast
460 thylakoid arrangements was useful to differentiate both lineages. These results suggest that
461 the thylakoid chloroplast arrangement is a key complement to the pyrenoid structure for
462 *Trebouxia* species characterisation.

463 In *F. soredians* a single *Trebouxia* lineage was identified both in the thallus and in
464 isolated culture (F1) (Fig.6, Supplementary Data Fig. S7). This result matched with molecular
465 data obtained by Sanger sequencing and 454-pyrosequencing.

466 In conclusion, to our knowledge this is the most comprehensive study combining in a
467 complementary approach molecular and ultrastructural data for the detection of intrathalline
468 *Trebouxia* lineages. This multi-tool analysis performs well when the coexistence of several
469 phycobionts inside a single thallus is expected. Comparative ultrastructural analyses of
470 phycobionts in the symbiotic state or isolated in culture could be performed because key
471 taxonomic features remain invariant enough within lichen thalli and in culture, allowing the
472 identification of each unique algal lineage, and therefore the differentiation of co-occurring
473 species. We are starting to shed light on questions that were previously unsolved (Catalá *et al*.
474 2015) due to the inability to link ultrastructural and molecular results. Moreover, we are
475 gaining a better understanding of how phycobiont diversity may play a role in the functioning
476 and development of lichen thalli or in the ability to cope with changing environmental
477 conditions.

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FIGURE LEGEND

Fig. 1. Scheme of experimental design followed in this study: two thalli of *Circinaria hispida* and *Flavoparmelia soredians* were first rehydrated for 24 hours and washed with sterile water. Several parts of this thallus were randomly selected and mixed (Pools A-D). **Sample A**: DNA extraction and nrITS DNA PCR to identify the mycobiont and the primary phycobiont by Sanger sequencing. **Sample B**: Phycobionts isolation following Gasulla *et al*. (2010) protocol. Fast microalgae identification: direct PCR were performed from well-developed colonies selected under the stereo-microscope. This procedure was done by using a sterile toothpick to pick the colony, and introducing it into the PCR mixture. Subsequently, the colonies were propagated directly by introducing this toothpick into liquid medium (BBM). To check the subculture, a second PCR was performed. Transmission electron microscopy examinations

(TEM) from selected culture. **Sample C**: 454-pyrosequencing. **Sample D**: TEM from thallus and comparative analysis with TEM from the isolated phycobionts. **AB**: From the culture strains sequenced in sample B, specific PCR primers were designed to detect different lineages using the DNA template from pool A.

Fig. 2. *Circinaria hispida* phycobiont diversity by Sanger sequencing and isolation. Unrooted nrITS DNA gene tree representing 72 *Trebouxia* sequences, including 24 nrITS *Trebouxia* species from SAG, UTEX and *Trebouxia* sp. TR9, and 5 OTUs described by Leavitt *et al.* (2015) retrieved from the GenBank. Values at branches refer to BI posterior probabilities ≥ to 0.9 and ML bootstrap values, respectively. Branches with a statistical support ≥ 90 % in both analyses are indicated in the tree. Underlined sequence refers to the primary detected phycobiont from the thallus, ○ designates the sequences obtained from the thallus by specific primers PCR, * designates OTUs that strongly fitted with our sequences. Four major clades described by Helms (2003) are indicated (Clade A, G, I, S). The ABGD program identified the four OTUs: T1, T2, T3 and T4.

Fig. 3. *Flavoparmelia soredians* phycobiont diversity by Sanger sequencing and isolation. Unrooted nrITS DNA gene tree representing 90 *Trebouxia* sequences including 22 nrITS *Trebouxia* species from SAG, UTEX and *Trebouxia* sp. TR9, and 2 OTUs described by Leavitt *et al.* (2015) were retrieved from the GenBank. Values at branches refer to BI posterior probabilities ≥ to 0.9 and ML bootstrap values, respectively. Branches with a statistical support ≥ 90 % in both analyses are indicated in the tree. Underlined sequence represents the primary detected phycobiont from the thallus, * designates the OTU that strongly fitted with

our sequences. Four major clades described by Helms (2003) are indicated (Clades A, G, I, S). The ABGD program identified a unique linage, F1.

Fig. 4. *Circinaria hispida* phycobionts in symbiotic and culture states by TEM. Comparative analyses between the photobionts isolated in cultures and those observed in the symbiotic state within the thallus A,B) T1; C,D) T2; E,F) T3; G,H) T4. Bars 600 nm, 800 nm, 1micron and 2 microns. Abbreviations: BT, Black tubules; Chl, Chloroplast; Pg, Pyrenoglobuli; Py, Pyrenoid; PyS, Small pyrenoidal structures; WT, White tubules.

Fig. 5. *Circinaria hispida* phycobionts coexistence by TEM. *Trebouxia* lineages T3 and T4. Bars 4 microns. Abbreviations: Hy, Hyphae; T3, lineage T3; T4, lineage T4; Py, Pyrenoid; PyS, Small pyrenoidal structures.

Fig. 6. *Flavoparmelia soredians* phycobiont in symbiotic and culture states by TEM: Comparative analyses. **A**: isolated lineage F1. **B**: intrathalline lineage. Bar 1 and 1.5 micron. Abbreviations: Chl, Chloroplast; Hy, Hyphae; Pg, Pyrenoglobuli; Py, Pyrenoid; T, Tubules.

SUPPLEMENTARY DATA

Supplementary Data Table S1. *Circinaria hispida* and *Flavoparmelia soredians*: Lichen mycobionts associated with *Trebouxia* T1- T4 and F1 lineages based on > 98% sequence similarity detected by Leavitt *et al.* (2015) and Voytsekhovich and Beck, (2015).

	Lichen mycobionts associated with *Trebouxia* lineages detected in *Circinaria hispida* and *Flavoparmelia soredians* based on > 98% sequence similarity
T1	*Parmelia*, *Protoparmeliopsis*, *Montanelia*, *Xanthoparmelia*, *Lecanora, Candelariella vitellina*, *Circinaria contorta*, *Diploschistes diacapsis*, *Porpidia crustulata*
T2	-
T3	*Protoparmeliopsis*, *Xanthoparmelia*, *Gyalolechia*, *Fulgensia*, *Lecidea*, *Lecidella*, *Psora*
T4	*Aspicilia, Protoparmeliopsis*, *Rhizoplaca*, *Xanthoparmelia*, *Buellia*, *Fulgensia*, *Lecidella*, *Peltigera*, *Psora*, *Squamarina, "Aspicilia desertorum", Rusavskia papillifera*
F1	*Melanohalea, Punctelia, Flavoparmelia, Physcia, Teloschistes, Xanthoria*

Supplementary Data Figure S1. *Circinaria hispida* phycobiont diversity by 454-pyrosequencing: Rooted nrITS DNA gene tree representing 21 *Trebouxia* sequences including representative sequences from each *Trebouxia* lineage detected (T1-T4), and a selection of nrITS *Trebouxia* species from SAG, UTEX and *Trebouxia* sp. TR9 retrieved from the GenBank. Pyrosequencing consensus sequences were encoded as: OTUcode_number of sequences. Values at branches refer to BI posterior probabilities and ML bootstrap values, respectively.

712

713 **Supplementary Data Figure S2.** *Flavoparmelia soredians* phycobiont diversity by 454-
714 pyrosequencing: Rooted nrITS DNA gene tree representing 11 *Trebouxia* sequences including
715 representative sequences from the *Trebouxia* lineage detected (F1), and a selection of nrITS
716 *Trebouxia* species from SAG, UTEX and *Trebouxia* sp. TR9 retrieved from the GenBank.
717 Pyrosequencing consensus sequences were encoded as: OTUcode_number of sequences.
718 Values at branches refer to BI posterior probabilities and ML bootstrap values, respectively.

719

720 **Supplementary Data Figure S3.** Pyrenoid detail from *Trebouxia* T1-type phycobionts in
721 symbiotic and culture states by TEM. Comparative analyses. **A-C-E-G**: isolated cultures T1.
722 **B-D-F-H**: intrathalline T1. Bars 300 nm and 400 nm. Abbreviations: Chl, Chloroplast; Pg,
723 Pyrenoglobuli.

724

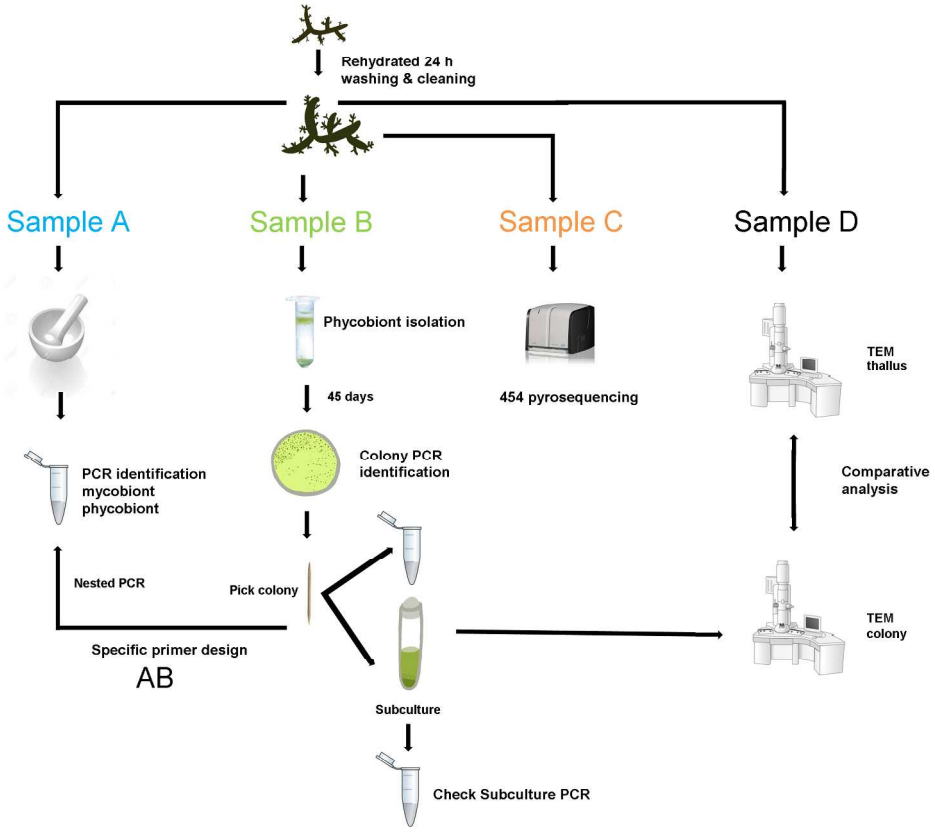
725 **Supplementary Data Figure S4.** Pyrenoid detail from *Trebouxia* T2-type phycobionts in
726 symbiotic and culture states by TEM. Comparative analyses. **A-C-E-G**: isolated cultures T2.
727 **B-D-F-H**: intrathalline T2. Bars 400 nm, 500 nm and 700 nm. Abbreviations: BT, Black
728 tubules; Chl, Chloroplast; WT, White tubules.

729

730 **Supplementary Data Figure S5.** Pyrenoid detail from *Trebouxia* T3-type phycobionts in
731 symbiotic and culture states by TEM. Comparative analyses. **A-C-E-G**: isolated cultures T3.
732 **B-D-F-H**: intrathalline T3. Bars 300 nm, 400 nm, 500 nm and 600 nm. Abbreviations: Chl,
733 Chloroplast; PyS, Small pyrenoidal structures.

734

735 **Supplementary Data Figure S6.** Pyrenoid detail from *Trebouxia* T4-type phycobionts in
736 symbiotic and culture states by TEM. Comparative analyses. **A-C-E-G**: isolated cultures T4.
737 **B-D-F-H**: intrathalline T4. Bars 300 nm, 500 nm and 600 nm. Abbreviations: Chl,
738 Chloroplast; Pg, Pyrenoglobuli; WT, White tubules.
739
740 **Supplementary Data Figure S7.** Pyrenoid detail from *Trebouxia* F1-type phycobionts in
741 symbiotic and culture states by TEM. Comparative analyses. **A-C-E-G**: isolated cultures F1.
742 **B-D-F-H**: intrathalline F1. Bars 400 nm, 500 nm and 600 nm. Abbreviations: Chl,
743 Chloroplast; Pg, Pyrenoglobuli; T, Tubules.
744



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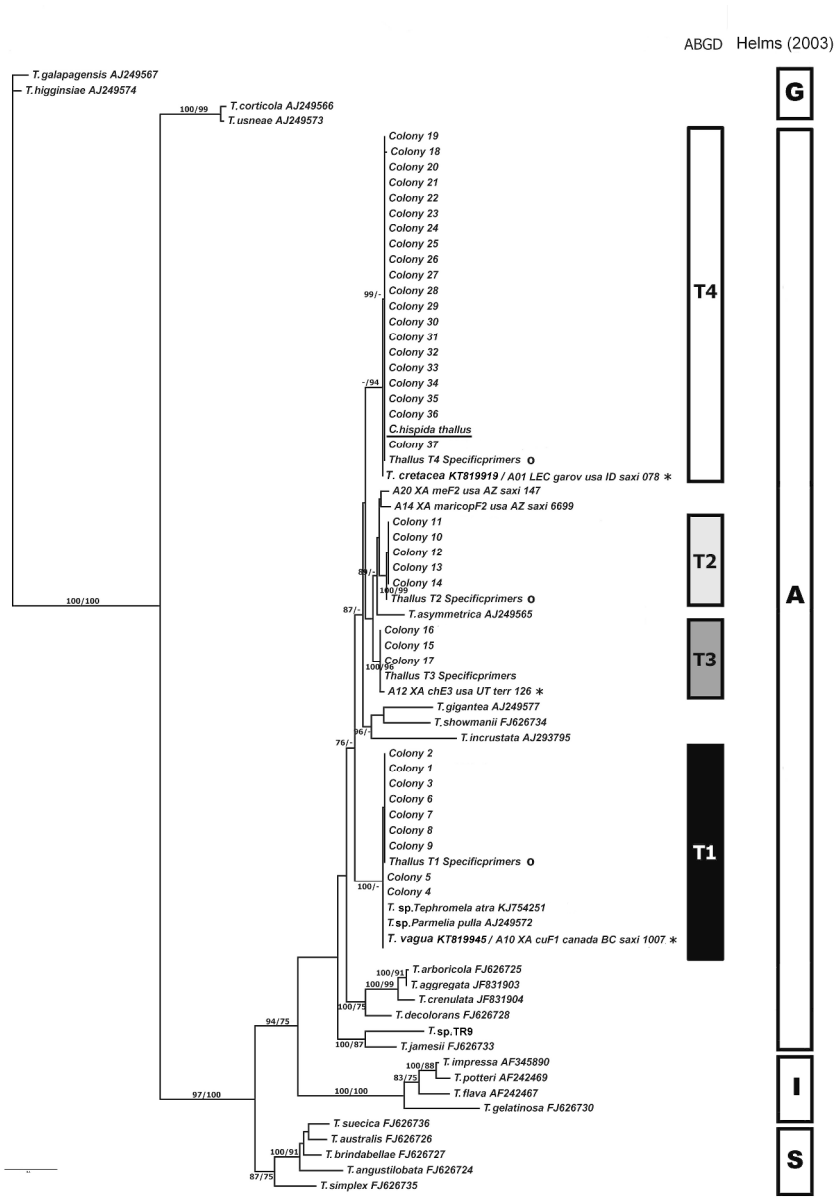

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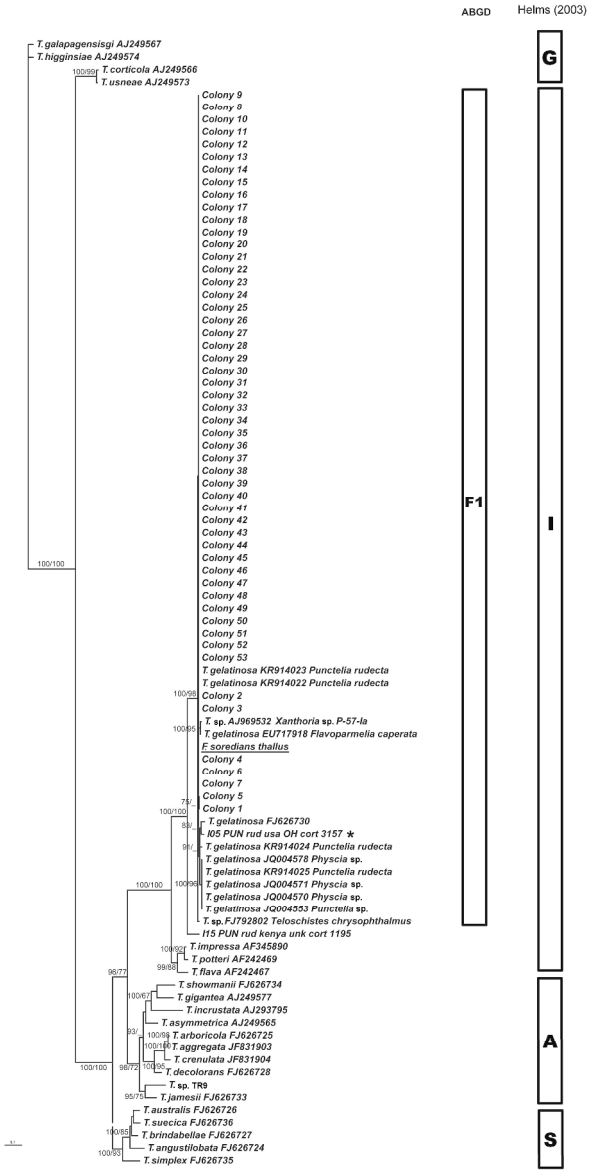

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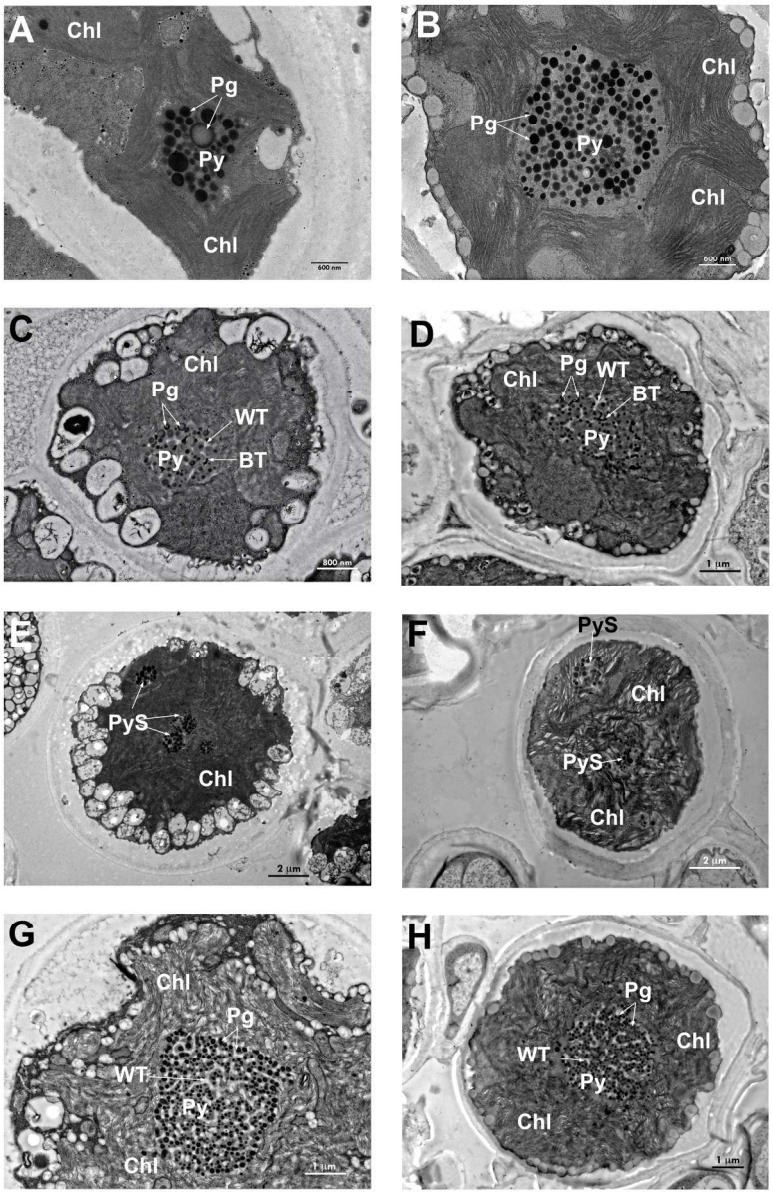

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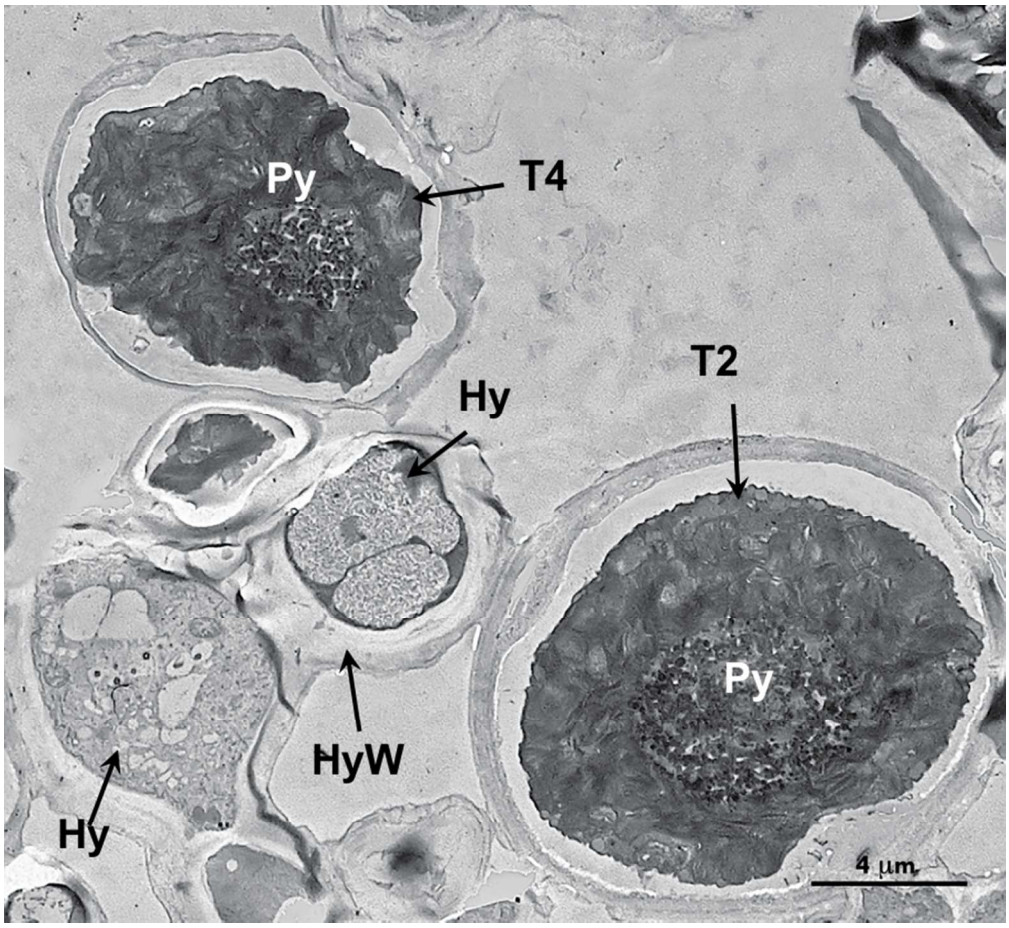

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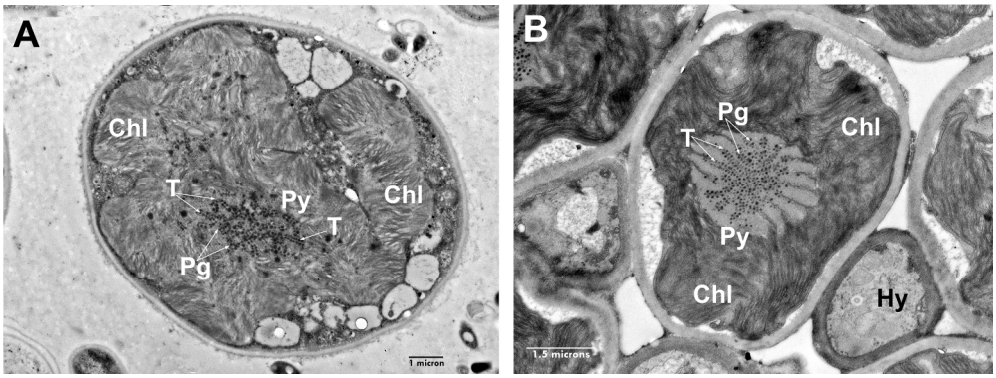

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