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Untangling the hidden intrathalline microalgal diversity in *Parmotrema pseudotinctorum*: *Trebouxia crespoana* sp. nov.

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Abstract: Intrathalline phycobiont diversity was investigated in a rosette-forming lichen, *Parmotrema pseudotinctorum*, using a combination of Sanger sequencing, 454-pyrosequencing, conventional light and confocal microscopy, and transmission electron microscopy. A total of 39 thalli sampled in five Canary Island populations were investigated. Three novel lineages of lichen phycobionts were detected, all being inferred within the *Trebouxia* clade G. The most abundant phycobiont lineage, occurring in all lichen populations investigated, is described here as *Trebouxia crespoana* sp. nov. This species produces spherical to pyriform cells possessing a crenulate chloroplast with lobes elongated at their ends, and one *corticola*-type pyrenoid with very thin, unbranched tubules of curved profile. *Trebouxia crespoana* is clearly distinguished from all other *Trebouxia* species by a characteristic cap-like cell wall thickening produced on one side of vegetative cells, and the larger size of vegetative cells that reach 21(–26) µm in diameter.

Key words: 454-pyrosequencing, Canary Islands, lichens, morphology, phycobionts, ultrastructure

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Introduction

Parmotrema pseudotinctorum (des Abb.) Hale is a rosette-forming foliose lichen which colonizes large areas on volcanic rock surfaces in arid or very dry and warm areas in the Canary Islands, mainly in the infra- and thermo-Mediterranean bioclimatic belts, surrounded by crassicaule or xeric Mediterranean vegetation (Molins *et al.* 2013). In some localities, even under extreme environmental conditions, this lichen can grow abundantly giving a distinctive appearance to the general landscape.

Recently, Roca-Valiente *et al.* (2013) studied the mycobiont using molecular phylogeny combined with morphological features, resolving the taxonomic identity of this species with regard to the closely related *Parmotrema tinctorum* (Delise ex Nyl.) Hale, which was occasionally considered to be a synonym. *Parmotrema pseudotinctorum* seems to be restricted to the Canary Islands, Africa, the Cape Verde Islands and India, the only place where it grows as an epiphyte on bark.

Thallus anatomy and ultrastructure were described by Molins *et al.* (2013), who detected the presence of a characteristic biofilm of bacteria that covers the underside of the thallus. In populations from Tenerife and Lanzarote, a collection of bacterial strains isolated from this lichen have been characterized physiologically and metabolically (Gimeno *et al.* 2016). The results revealed that most bacterial strains were capable of producing proteases and nucleases as well as solubilizing polysaccharides and phosphate. In addition, siderophores and more than 75% of fixed nitrogen were produced as well as some auxins and they formed biofilms.

These activities may have an important role in the functioning of the symbiosis and undoubtedly have biotechnological applications.

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Over the last ten years, several studies have focused on the phycobiont inventory of the closely related *P. tinctorum* and *P. pseudotinctorum* lichens. These investigations revealed the existence of considerable phycobiont diversity within the *Trebouxia* clade G *sensu* Helms (2003). First, Ohmura *et al.* (2006) detected a total of six phycobiont lineages within *P. tinctorum* thalli sampled in the Shimizu district of Japan. Later, Mansournia *et al.* (2012) investigated the population heterogeneity of phycobionts growing within *P. tinctorum* in the Chiba Prefecture, Japan. They revealed the presence of four phycobiont lineages, two of them being identical to the lineages already detected by Ohmura *et al.* (2006). Finally, Molins *et al.* (2013) identified three phycobiont lineages in their investigation of *P. pseudotinctorum* lichens growing on the Canary Islands, Spain. Interestingly, none of these lineages were identical to any of the previously sequenced *P. tinctorum* phycobionts (Ohmura *et al.* 2006; Mansouria *et al.* 2012). In addition, all three previously-mentioned studies detected the presence of multiple phycobiont lineages within single *Parmotrema* thalli.

In general, none of the previously identified lineages of *P. tinctorum*/*P. pseudotinctorum* phycobionts could be assigned to previously described *Trebouxia* species, emphasizing our poor knowledge of the diversity, evolution and taxonomy of the phycobiont genus *Trebouxia*. However, such knowledge is essential for our understanding of the functioning and complexity of lichen symbiotic interactions. In this paper, we therefore aimed to obtain a more complete picture of the intrathalline phycobiont diversity in *P. pseudotinctorum*, and to morphologically characterize and formally describe the most abundant phycobiont associated with this lichen. Here, we describe this phycobiont as *Trebouxia crespoana* sp. nov.

Materials and Methods

Lichen material

Five representative populations of *Parmotrema pseudotinctorum* collected from the Canary Islands were included in the analyses. Samples from San Sebastián (La Gomera, LG) were collected on old basalt rocks. The samples from Los Cancajos and Breña Baja (La Palma, LP) were collected on basalts from the flow of the "Cumbre Vieja" volcano. Lichen thalli from Jameos del Agua (Lanzarote, LZ) were collected on subalkaline basalt rocks originating from the sub-recent flow of the La Corona volcano, and samples from Buenavista (Tenerife, TF) were collected on old basalts. The samples were dried and stored at −20 °C until processing. Each lichen specimen used in this study was encoded as indicated in Supplementary Table S1 (available online).

Sample handling

Lichen thalli were examined under a stereomicroscope to remove surface contamination (e.g. bark, sand, mosses, fragments of other lichen species, or infection by lichenicolous fungi). Lichen thalli were sterilized by sequential immersion in 96% ethanol (10 s), 0·5% NaOCl (2 min) and 70% ethanol (2 min) (Arnold *et al.* 2009). Fragments from different parts of the thallus (centre, mid-point and edge) were randomly excised and pooled together. The mycobiont and the primary phycobiont were identified by Sanger sequencing, and the Los Cancajos PAL 4 sample was also analysed by 454-pyrosequencing. The ultrastructure of the phycobionts was characterized by transmission electron microscopy (TEM) for the Breña Baja 2 and 5 samples. The Los Cancajos 10 sample was used to isolate the phycobiont in the culture for its investigation by conventional light (LM) and confocal microscopy (CM).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated and purified using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Two algal loci were amplified; a region of the chloroplast LSU rDNA gene using the algal specific primers 23SU1 and 23SU2 (del Campo *et al.* 2010), and the ITS rDNA using the primer pair Parm_ITS_Phyco_F (5´- TGA TTC TAT CGT GCC AAC ACC G -3´) and Parm_ITS_Phyco_R (5´- GAT ATG CTT AAG TTC AGC GGG TG -3´) designed in our laboratory based on the previous ITS1-ITS2 alignments obtained in Molins *et al.* (2013). Fungal ITS rDNA was amplified using the primer pair Parm_ITS_Myco_F (5´- TGA GAG AGG GGC TTC GCG CTC C -3´) and Parm_ITS_Myco_ R (5´- ATC CGA GGT CAA TAT TGG AAG CA- 3´) designed for this study. PCR reactions were performed in 50 µl using 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 consisted of an initial denaturation at 94 °C for 2 min and 30 cycles at 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. Amplifications were carried out on a 96-well SensoQuest Labcycler (Progen Scientific Ltd., South Yorkshire, UK). The PCR products were visualized on 2% agarose gels and purified using the Gel Band Purification Kit (GE Healthcare Life Science, Buckinghamshire, UK). The amplified PCR

products were sequenced with an ABI 3100 Genetic Analyzer using the ABI BigDyeTerminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA).

Sequence analyses

The newly determined algal nuclear ITS rDNA and chloroplast LSU rDNA sequences were aligned with the selection of *Trebouxia* sequences from the GenBank database, with emphasis given to the authentic strains of *Trebouxia* species. The alignment was then supplemented by a number of sequences belonging to *Trebouxia* clade G, including all new lineages identified by Ohmura *et al.* (2006), Mansournia *et al.* (2012) and Molins *et al.* (2013). The sequences were aligned using MAFFT v.6 software (Katoh *et al.* 2002) under the Q-INS-I strategy, N treated as a wildcard and checked for obvious sequencing errors. The alignment of ITS rDNA sequences was improved by eliminating the ambiguously aligned regions using the program Gblocks v.0.91b (Castresana 2000). The two loci were concatenated, yielding an alignment of 1477 characters. The identical sequences were merged, resulting in the final matrix containing 54 ITS rDNA and 26 LSU rDNA sequences. For each locus, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al.* 2012). This BIC-based model selection procedure selected the GTR + I + Γ and TPM3uf + I + Γ models for the ITS and LSU rDNA regions, respectively.

The phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes v.3.2.6 (Ronquist *et al.* 2012), carried out on partitioned datasets using the GTR + I + Γ substitution models for both partitions (the TPM3uf model was not implemented). Two parallel MCMC runs were carried out for six million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0·0024. Finally, the burn-in values were determined using the 'sump' command. Bootstrap analyses were performed by maximum likelihood (ML) using GARLI v.2.01 (Zwickl 2006). ML analysis consisted of rapid heuristic searches (100 pseudoreplicates) using automatic termination (genthreshfortopoterm command set to 100 000). The analysis was performed on partitioned datasets using the different substitution models selected by jModelTest 2.1.4. All analyses were run on the CIPRES Science Gateway v.3.3 web portal (Miller *et al.* 2010).

454-pyrosequencing and phylogenetic analyses

A first RT-PCR (RT-PCR I) and a first PCR (PCR I) were performed using the genomic DNA from the Los Cancajos PAL 4 sample as a template and nr-SSU-1780/5.8S 2R primers (Moya *et al.* 2017). The number of cycles of PCR I (19 cycles) was determined by the average Ct (cycle threshold) of the RT-PCR I. We then performed a second RT-PCR (RT-PCR II) and a second PCR (PCR II) using 1 µl of the PCR I as 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 (7 cycles) was determined by the average Ct from the RT-PCR II. The RT-PCRs and PCRs were performed and purified as previously described in Moya *et al.* (2017). Algal ITS rDNA 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 as described in Moya *et al.* (2017) and clustered based on 99% score coverage threshold and 90% length coverage threshold (-S 99 -L 0·9) criteria. The consensus sequences of the OTUs were identified using the BLAST tool in the GenBank database (Altschul *et al.* 1990).

Ultrastructural examinations

Transmission electron microscopy (TEM) examinations were performed on samples 2 and 5 from Breña Baja. For TEM, the cells were fixed in 2% Karnovsky fixative for 12 h at 4 °C and washed three times for 15 min with 0·01 M PBS (pH 7·4) 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 series of ethanol solutions, starting at 50% and increasing to 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 (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, using the "SynapTek Grid Staining Kit" (http://www.emsdiasum.com/microscopy/technical/datasheet/71175.aspx). The sections were observed with a JEOL JEM-1010 (80 kV) electron microscope equipped with a MegaView III digital camera and "AnalySIS" image acquisition software. TEM examinations were carried out at the SCSIE Service of the University of Valencia.

Isolation and propagation of microalgae

Phycobionts were isolated from sample no. 10 from Los Cancajos using the micromethod described by Gasulla *et al.* (2010). Samples were homogenized 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®. The algal suspension was diluted with sterile water and 10 µl was streaked onto sterile 1·5% agar Bold's Basal Media Petri dishes (BBM) (Bold 1949; Bischoff & Bold 1963). The isolated algae were maintained at 15 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) with a 12 h photoperiod at 21°C. To

obtain unialgal cultures, small populations of phycobionts were transferred onto the fresh BBM agar slants and incubated accordingly.

Microscopy

The morphology of the isolated phycobiont strain was investigated by both conventional light (LM) and confocal (CM) microscopy. LM observations were performed using an Olympus BX51 microscope equipped with differential interference contrast. Micrographs were taken with an attached Canon EOS 700D camera. For CM, a Leica TCS SP2 laser scanning confocal microscope equipped with an argon-krypton laser was used. We applied a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. A series of optical sections through chloroplasts was captured and used for 3-dimensional reconstruction of their morphology. The chloroplast reconstructions were produced by the ImageJ v.1.34p program (Abramoff *et al.* 2004), using the "Volume viewer" plugin. Zoospore formation was induced by transferring the culture in different ontogenetic stages to distilled water.

Results

Trebouxia crespoana Barreno, Molins, Moya & Škaloud sp. nov.

AlgaeBase ID: Aea4fa8abaec4fb8a

Differing from other *Trebouxia* species by the formation of pyriform cells bearing local cell wall thickening and by the order of the nucleotides in ITS rDNA sequences.

Type: Spain, Canary Islands, La Palma, Breña Baja, flow of "Cumbre Vieja" volcano rocks, 28°38′33″N, 17°45′30″W, phycobiont in *Parmotrema pseudotinctorum*, 11 December 2016, *A. Santos-Guerra* (MAF-Lich 21323—holotype; MA-Lich 19165, VAL_Lich 30719—isotypes).

(Figs 1 & 2)

Cells are solitary, usually spherical, rarely oval and pyriform, 11–21(–26) µm diam. Cell wall is usually thin. Sometimes a characteristic cap-like cell wall thickening is produced on one side of vegetative cells. The cells possess a central crenulate chloroplast with several lobes elongated at their ends; only rarely are two crenulate chloroplasts formed within a single cell. The chloroplast contains one spherical or irregularly elongated pyrenoid covered by a layer of a few large, arched starch grains. Pyrenoid is *corticola*-type with very thin, unbranched tubules of curved profile, and pyrenoglobuli developed in the closest parts of the chloroplast stroma. In mature and old cells, the pyrenoid is usually indistinct. Asexual reproduction involves the formation of autospores and zoospores. The autosporangia of irregular shape usually contain 4–16 autospores, tightly appressed to each other. Zoospores are biflagellate, drop-shaped or oval, 5·5–6·0 µm long and 4·0–4·5 µm wide, with posterior chloroplast. Sexual reproduction was not observed.

Reference strains. CAUP H 1019 (Culture Collection of Algae of Charles University in Prague), Strain 132 (E. Barreno's personal collection at the University of Valencia).

Etymology. The specific epithet refers to the Canarian Professor of Botany Dr Ana Crespo. She has developed lichen studies in Spain over the last quarter of the 20th century and pioneered studies of phylogenetic systematics with molecular markers; especially innovative were her analyses of *Parmeliaceae* s. a.

Ecology and distribution. *Trebouxia crespoana* was found only in symbiosis with the foliose lichen *Parmotrema pseudotinctorum*, growing as saxicolous on diverse types of volcanic rock on several islands of the Canarian archipelago. This rosette-forming lichen is prevalent and yields large biomass in the infra-Mediterranean arid bioclimatic belt but can reach the thermo-Mediterranean subhumid belt in the driest areas of the laurel forests. So far, *T. crespoana* is known only from the Canary Islands.

Morphology of cultured *Trebouxia crespoana*

Young cells were spherical, containing one axial chloroplast with several lobes and a distinct pyrenoid (Fig. 1A). Mature vegetative cells were up to 21(–26) µm in diameter, usually spherical in shape (Fig. 1B) but oval and pyriform cells were also observed. The cell wall was usually thin; however, in some

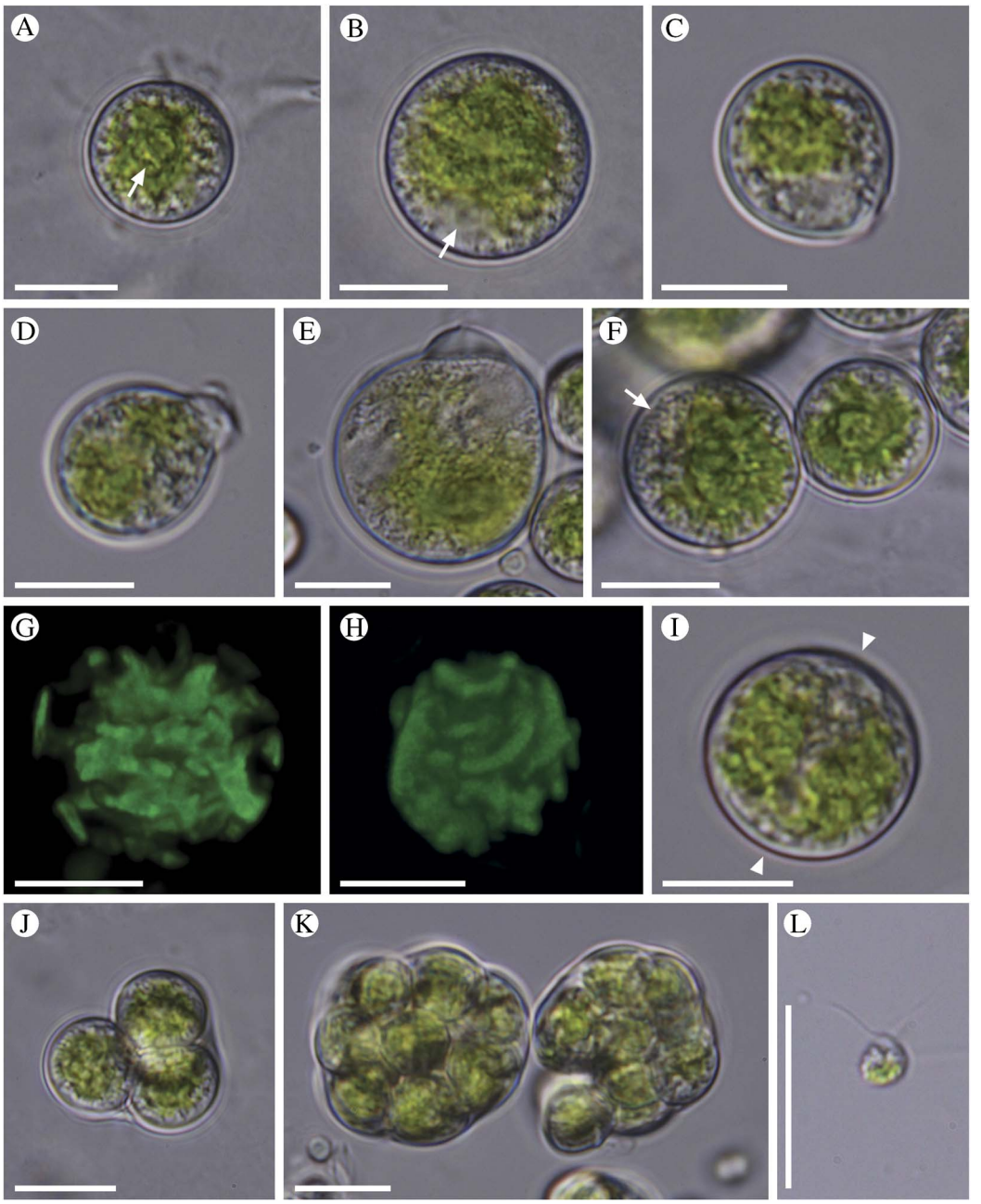

FIG. 1. Morphology of *Trebouxia crespoana* sp. nov. A, young cell with a prominent pyrenoid (arrow); B, mature vegetative cell (nucleus labelled with an arrow); C, a pyriform cell with papilla-like cell wall thickening; D, cell with a prominent cell wall thickening; E, old cell with a distinct cell wall bulge; F, cells with a central, crenulate chloroplast (peripheral vesicles labelled with an arrow); G, reconstruction of chloroplast in a mature cell; H, reconstruction of a chloroplast surface, note the elongated ends of chloroplast lobes; I, a cell with two adjacent chloroplasts (space between the chloroplasts is marked with arrowheads); J, small autosporangium with 4 autospores; K, irregularly-shaped autosporangia; L, a zoospore. Scales = 10 μm.

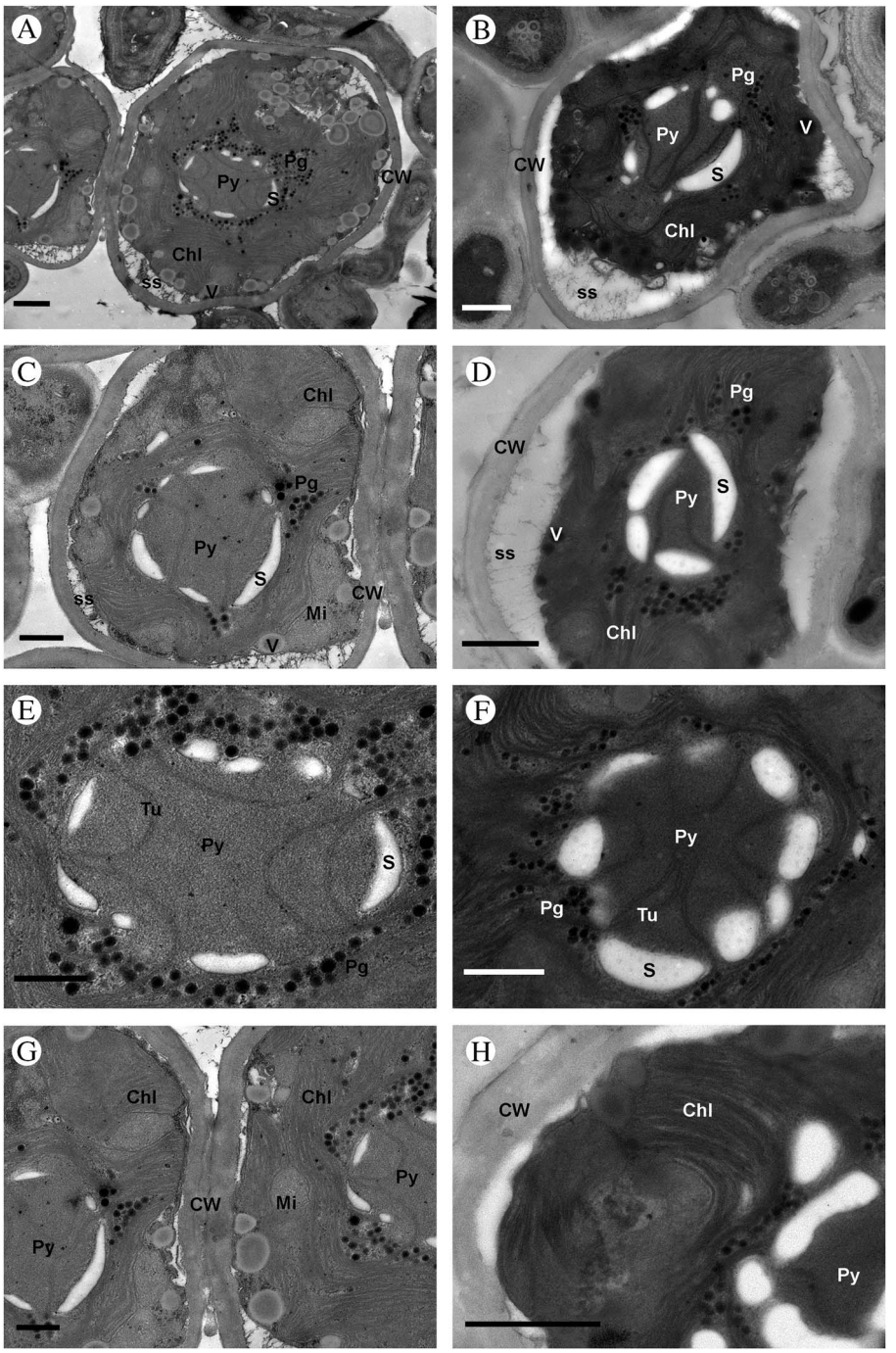

FIG. 2. Ultrastructure of *Trebouxia crespoana* sp. nov. A–D, *T. crespoana* cells within thallus; E & F, details of pyrenoid; G & H, detail of the cell wall and thylakoid arrangement. Abbreviations: Chl (chloroplast), CW (cell wall), Mi (mitochondrion), Pg (pyrenoglobuli), Py (pyrenoid), S (starch grain), SS (secretory space), Tu (tubules) and V (vesicles). Scales: A & H = 1 μm; B, D & F = 800 nm; C, E & G = 600 nm.

cells a local cell wall thickening had developed on one side of the vegetative cells. First, a small, papilla-like cell wall thickening occurred, followed by the formation of a distinct cell wall bulge (Fig. 1C). We did not observe any cell wall ruptures or other processes following the cell wall bulging; instead, the cell wall thickening was retained in mature and old cells (Fig. 1D & E). The thickenings measured 0·5–3·5 µm. *T. crespoana* possesses a central crenulate chloroplast with several lobes spreading toward the cell periphery (Fig. 1F & G). The lobes were notably elongated at their ends (Fig. 1H). Rarely, two adjacent crenulate chloroplasts were observed (Fig. 1I). The chloroplast contained one centrally positioned pyrenoid (Fig. 1A) surrounded by starch grains (Fig. 1F). The pyrenoid was clearly visible in young cells but indistinct in mature and old cells. The cells were uninucleate, with a lateral nucleus positioned between the chloroplast lobes (Fig. 1B). Usually a large number of small peripheral vesicles were observed underneath the cell wall (Fig. 1F). The cells reproduced only asexually, by the formation of autospores and zoospores. The autosporangia were first spherical, later irregularly-shaped and deformed by the growing autospores, up to 24 µm in length, usually containing 4–16 spherical autospores (Fig. 1J & K). When released, they measured 5·5–9·0 µm in diameter. Zoosporangia were spherical, containing an unspecified number of zoospores. Zoospores were biflagellate, teardrop-shaped or oval, 5·5–6·0 µm long and 4·0–4·5 µm wide, with posterior chloroplast (Fig. 1L).

Ultrastructure of lichenized *Trebouxia crespoana* (Fig. 2)

Lichenized cells were mostly oval to rounded, up to 7·8 µm in diameter (Figs 2A–C). The cell wall was thin, 0·18–0·33 µm wide (Figs 2C & D). The secretory space was evenly distributed except in the zones in contact with mycobiont haustoria (Fig. 2D). The interaction of phycobionts and the mycobiont was frequently made through type 2 haustoria *sensu* Honegger (1986). The centrally positioned chloroplast extends into dividing lobes showing a regular and dense distribution of the thylakoid membranes (Figs 2C & H) with some pyrenoglobules. The central pyrenoid was spherical or irregularly elongated, of the *corticola*-type *sensu* Friedl (1989), with very thin, curved, unbranched tubules (Figs 2E & F). Pyrenoglobuli developed primarily in the innermost parts of the chloroplast stroma (Fig. 2E). Starch grains were closely associated with the pyrenoid matrix, forming a starch sheath made up of a few large, arched plates adjacent to the pyrenoid (Figs 2E & F). The cells possess a few mitochondria, and in the peripheral zones numerous vesicles with a central electrodense granular content in a non-electrodense matrix (Figs 2C & G). These vesicles were clearly visible in cultured cells observed under light microscopy (Fig. 1F).

Mycobiont identification and the diversity of primary phycobionts

Morphological identification of investigated lichens was verified by BLAST searches of mycobiont ITS rDNA sequences. All 39 mycobiont sequences were closely related or identical to *P. pseudotinctorum* isolates molecularly characterized by Roca-Valiente *et al.* (2013).

Diversity of primary phycobionts was much higher in comparison to the mycobiont partners. All algal sequences were inferred within the *Trebouxia* clade G *sensu* Helms (2003). The majority of lichen thalli contained the new phycobiont lineage described here as *T. crespoana* sp. nov. (Fig. 3). This species was found in all lichen populations investigated and was previously reported by Molins *et al.* (2013) and Leavitt *et al.* (2015). Seven lichen thalli collected in the Buenavista and Los Cancajos populations contained a different algal lineage, sister to *T. crespoana* and *Trebouxia* OTU G04 *sensu* Leavitt *et al.* (2015). This lineage was previously reported in many Japanese *P. tinctorum* lichens collected by Ohmura *et al.* (2006) and Mansournia *et al.* (2012), and labelled as clade I and *T. corticola*, respectively. Finally, the primary phycobionts of three

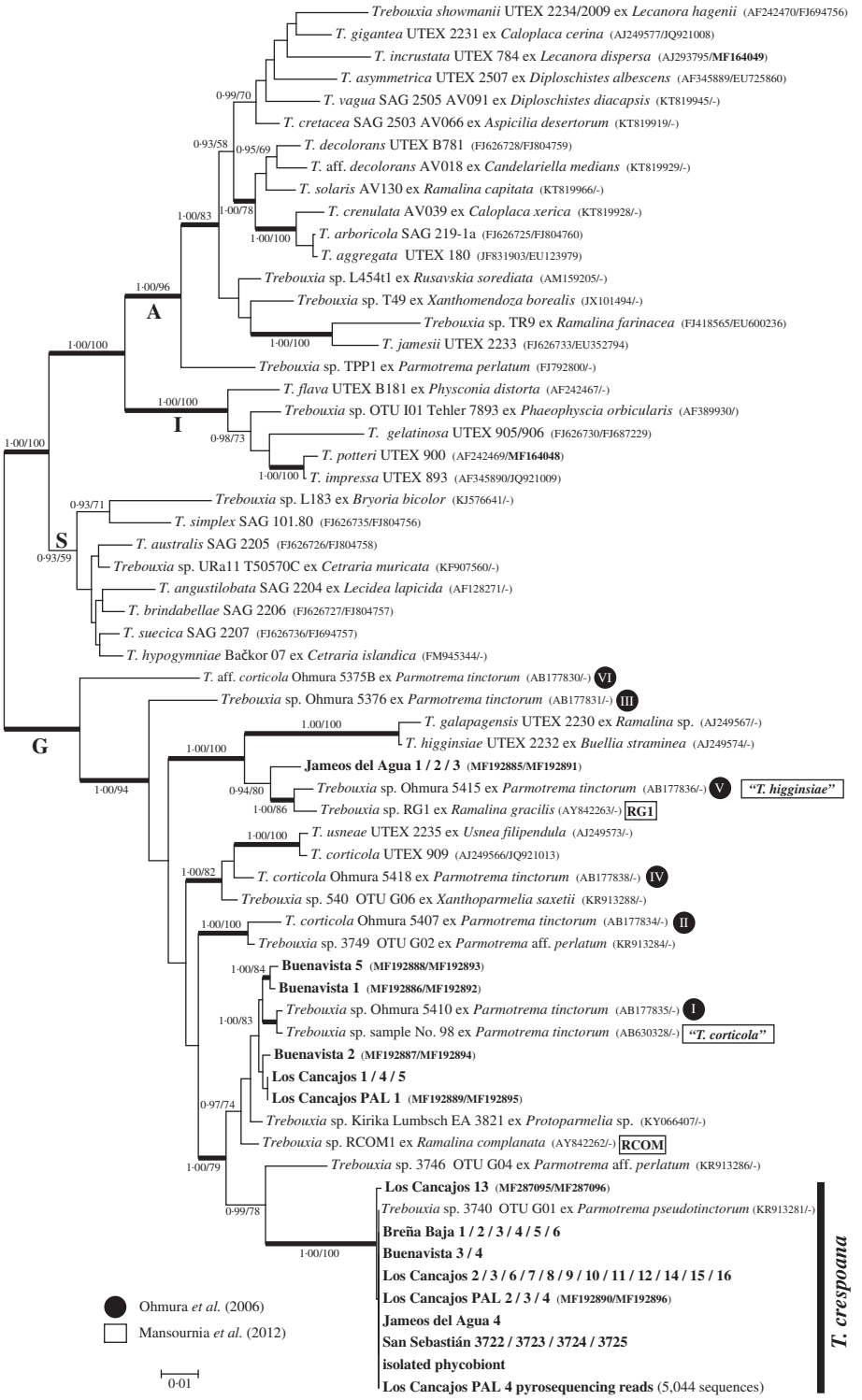

TABLE 1. *Taxonomic identification of the eight OTUs detected according to BLAST matches in GenBank and numbers of the corresponding sequences present in each OTU.*

Code	Filtered reads	BLAST match	Identity-Coverage
OTU 1	5044	*Trebouxia* sp. OTU G01 (KR913281)	79%-100%
OTU 2	6	*Trebouxia* sp. P-280IIaSc (AJ969583)	100%-100%
OTU 3	5	*Trebouxia asymmetrica* (KT819927)	100%-99%
OTU 4	5	*Trebouxia* sp. P-288-Ia (AJ969587)	100%-99%
OTU 5	3	*Trebouxia impressa* (KF907543)	100%-99%
OTU 6	3	*Myrmecia* sp. URa19 (KF907687)	93%-99%
OTU 7	2	*Trebouxia* sp. OTU G01 (KR913281)	79%-100%
OTU 8	2	*Asterochloris mediterranea* IO3 (AF345435)	100%-100%

lichens collected in the Jameos del Agua population were inferred in a lineage related to another *P. tinctorum* phycobiont labelled as clade V and *Trebouxia* RG1 by Ohmura *et al.* (2006) and Mansournia *et al.* (2012), respectively.

Phycobiont diversity by 454-pyrosequencing

Sequencing of ITS rDNA amplicons produced 5096 sequence reads for Los Cancajos PAL 4. Singleton reads (26) were filtered out. By clustering with a 99% similarity cut-off, eight OTUs were recognized: OTU1–OTU8 (Table 1). These OTUs were representative of three phycobiont genera: *Trebouxia*, *Asterochloris* and *Myrmecia*. The OTUs 1 and 7 matched with *T. crespoana* and with the primary phycobiont detected by Sanger sequencing in Los Cancajos PAL 4. OTU 3 fitted with *T. asymmetrica*. OTU 5 was related to *T. impressa* and OTUs 2 and 4 to *T. decolorans.* Significant matches were obtained with *Asterochloris mediterranea* (AF345435) and *Myrmecia* sp. (KF907687) for OTU 8 and OTU 6, respectively.

Discussion

Distinctness of *Trebouxia crespoana* sp. nov.

Trebouxia represents an extremely diverse and probably the most common genus of lichen phycobionts, occurring worldwide in thalli of numerous lichen fungal genera (Tschermak-Woess 1988; Miadlikowska *et al.* 2014). Traditionally, some *Trebouxia* species were exclusively delimited based on morphological features such as cell size and shape, chloroplast morphology, number and morphology of pyrenoids, dissociation of autospores, and zoospore morphology (Ettl & Gärtner 1995). However, our knowledge concerning overall *Trebouxia* species diversity has dramatically changed over the last two decades, since a number of molecular phylogenetic investigations revealed substantial hidden diversity within the genus (Kroken & Taylor 2000; Dahlkild *et al.* 2001; Blaha *et al.* 2006; Muggia *et al.* 2010, 2014; Sadowska-Des *et al.* 2014; Leavitt *et al.* 2016). Despite this, only a very small fraction of newly recognized lineages have been formally described as new species (Beck 2002; Voytsekhovich & Beck 2016). With the discrepancy between observed

FIG. 3. Phylogeny of the genus *Trebouxia* obtained by Bayesian inference of the concatenated nuclear ITS rDNA and chloroplast LSU rDNA sequences. Values at the nodes indicate statistical support estimated by MrBayes posterior-node probability (left of slash) and maximum-likelihood bootstrap (right of slash). Thick branches represent nodes receiving the highest posterior probability support (1·00). Newly sequenced strains and newly generated sequences are marked in bold. Identical sequences obtained from the same localities are shown in one row, along with the sampling codes. Clade affiliation *sensu* Ohmura *et al.* (2006) and Mansournia *et al.* (2012) is indicated next to the sequences by black circles and white rectangles, respectively. Four main *Trebouxia* clades (A, I, S, G) are indicated by letters on the backbone of the tree. Scale bar represents the expected number of substitutions per site.

diversity and described species in mind, we are here proposing a new species for the most common lineage of photobionts we detected during our investigation of *Parmotrema pseudotinctorum* thalli. Though *T. crespoana* represents a distinct and statistically well-supported lineage within *Trebouxia* clade G, a morphological comparison to all previously described *Trebouxia* species is necessary to avoid describing a junior synonym of an already described yet molecularly uncharacterized species. Morphologically, *T. crespoana* is well recognized by the formation of pyriform cells bearing local cell wall thickening (Fig. 1C–E). Such a feature has been observed only in *T. asymmetrica*, a symbiont described and isolated by Friedl & Gärtner (1989) from the lichen *Diploschistes albescens*. However, the cells of this species are much smaller and more ovoid than those of *T. crespoana*. In addition, the authentic strain of *T. asymmetrica* (UTEX 2507) has been sequenced by Piercey-Normore & DePriest (2001), showing its phylogenetic position within the *Trebouxia* clade A *sensu* Helms (2003), unrelated to all *P. pseudotinctorum* phycobionts.

Trebouxia crespoana matches with *Trebouxia* OTU G01, forms a well-supported clade together with *Trebouxia* OTU G04 *sensu* Leavitt *et al.* (2015) and a lineage comprising several phycobionts found in lichen thalli collected in the Buenavista (samples 1, 2, 5) and Los Cancajos (samples 1, 4, 5, PAL1) populations. Although the majority of these phycobionts were detected in *Parmotrema* lichens sampled in the Canary Islands, it is highly likely that both *Trebouxia* OTU G04 and the Buenavista-Los Cancajos lineage represent two distinct yet undescribed *Trebouxia* species. The genetic differences among these lineages and *T. crespoana* are almost as great as those between any sister *Trebouxia* species (Fig. 3). Indeed, *T. crespoana* and *Trebouxia* OTU G04 were already recognized as different species by Leavitt *et al.* (2015) using the ABGD delimitation method. In addition, several published studies demonstrated the strong ecological differentiation of closely related green-algal lineages, including lichen phycobionts (Peksa & Škaloud 2011; Moniz *et al.* 2012; Škaloud & Rindi 2013; Sadowska-Des *et al.* 2014; Malavasi *et al.* 2016; Ryšánek *et al.* 2016), indicating these lineages represent distinct cryptic species. Although we did not collect any ecological data with the samples collected, we are quite sure that applying a broad species concept, grouping all Canary Island phycobionts into a single taxon *T. crespoana*, would lead to masking the real species diversity within the genus *Trebouxia*. Our data rather demonstrate that the vast majority of aerophytic and symbiotic green algae still remain undescribed, even though they may represent the most common and ecologically important organisms in certain areas. The Canary Islands are a suitable example of such a phenomenon (Molins *et al.* 2013; Vančurová *et al.* 2015).

Investigation of intrathalline phycobiont diversity

Although 454-pyrosequencing has already been surpassed by other high throughput sequencing (HTS) approaches, it still represents a powerful and complementary approach to the traditional Sanger sequencing. HTS approaches supply information to evaluate species diversity at diverse taxonomic levels and provide much higher resolution to reveal the multiplicity of microalgae associated with the lichen thallus. The coexistence of multiple microalgae inside a single lichen thallus has been reported in some 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, 2018; Leavitt *et al.* 2015; Moya *et al.* 2017), whereas other lichens can be considered as highly specific, accepting only one single algal lineage in their thallus as a partner (Piercey-Normore & DePriest 2001; Beck *et al.* 2002; Yahr *et al.* 2004; Doering & Piercey-Normore 2009; Peksa & Škaloud 2011; Piercey-Normore & Deduke 2011; Molins *et al.* 2018). The sample of *P. pseudotinctorum* analyzed by 454-pyrosequencing in the present work showed only one *Trebouxia* sp. associated with the thallus (*Trebouxia* sp. OTU G01: 5044 reads). The remaining seven OTUs detected

represent between 0·03 and–0·1 % of the sequence reads and cannot be considered as strictly intrathalline.

These huge differences in species abundances raise questions about the levels of specificity and the influence of ecological settings in the association of lichenized fungi with certain algae available in the substratum pool (Peksa & Škaloud 2011). Sterilization of lichen material is essential in avoiding surface contamination, but OTUs representing <0·5% can be considered as the possible epithalline algal fraction (Moya *et al*. 2017).

Ultrastructural investigations

In this study, TEM was used to characterize the ultrastructure of *T. crespoana* cells isolated from *P. pseudotinctorum* (Fig. 2). The observations were made on different samples and no differences were found between them. Cells showed a characteristic *corticola*-type pyrenoid (Friedl 1989) with the absence of pyrenoglobuli associated with the matrix thylakoids, and starch grains forming a starch sheath. Besides molecular techniques, phycobiont identity should be validated through microscopic examination including TEM (Muggia *et al*. 2010; Casano *et al*. 2011; Molins *et al*. 2013, 2018; Moya *et al*. 2015; Catalá *et al*. 2016). Although *T. crespoana* represents a distinct and well-supported lineage within *Trebouxia* clade G, the cells showed the same pyrenoid type as *T. corticola*, *T. galapagensis*, *T. higginsiae* and *T. usneae* (Friedl 1989). Ultrastructural traits of pyrenoids from cultured phycobionts have been traditionally used to characterize *Trebouxia* species (Friedl 1989). However, our up-to-date knowledge of *Trebouxia* diversity has expanded and the original classification proposed by Friedl (1989) is in need of revision. This will allow ongoing and future studies to better delimit heterogeneous lineages in *Trebouxia*.

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Supplementary Material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S0024282918000208

References

Abramoff, M. D., Magalhaes, P. J. & Ram, S. J. (2004) Image processing with ImageJ. *Biophotonics International* **11:** 36–42.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215:** 403–410.

Arnold, A. E., Miadlikowska, J., Higgins, K. L., Sarvate, S. D., Gugger, P., Way, A., Hofstetter, V., Kauff, F. & Lutzoni, F. (2009) A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? *Systematic Biology* **58:** 283–297.

Beck, A. (2002) *Selektivität der Symbionten schwermetalltoleranter Flechten*. Ph.D. thesis, Universität München.

Beck, A., Kasalicky, T. & Rambold, G. (2002) Myco-photobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. *New Phytologist* **153:** 317–326.

Bischoff, H. W. & Bold, H. C. (1963) Phycological Studies IV. Some soil algae from Enchanted Rock and related algal species. *University of Texas Publications* **6318:** 1–95.

Blaha, J., Baloch, E. & Grube, M. (2006) High phycobiont diversity in symbioses of the euryoecious lichen *Lecanora rupicola* (*Lecanoraceae*, Ascomycota). *Biological Journal of the Linnean Society* **88:** 283–293.

Bold, H. C. (1949) The morphology of *Chlamydomonas chlamydogama* sp. nov. *Bulletin of the Torrey Botanical Club* **76:** 101–108.

Casano, L. M., del Campo, E. M., García-Breijo, F. J., Reig-Armiñana, J., Gasulla, F., del Hoyo, A., Guéra, A. & Barreno, E. (2011) Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus competition? *Environmental Microbiology* **13:** 806–818.

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

Catalá, S., del Campo, E. M., Barreno, E., García-Breijo, F. J., Reig-Armiñana, J. & Casano, L. M. (2016) Coordinated ultrastructural and phylogenomic analyses shed light on the hidden phycobiont diversity of *Trebouxia* microalgae in *Ramalina fraxinea*. *Molecular Phylogenetics and Evolution* **94:** 765–777.

Dahlkild, Å., Källersjö, M., Lohtander, K. & Tehler, A. (2001) Photobiont diversity in the *Physciaceae* (*Lecanorales*). *Bryologist* **104:** 527–536.

Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

del Campo, E. M., Casano, L. M., Gasulla, F. & Barreno, E. (2010) Suitability of chloroplast LSU rDNA and its diverse group I introns for species recognition and phylogenetic analyses of lichen-forming *Trebouxia* algae. *Molecular Phylogenetics and Evolution* **54:** 437–444.

Doering, M. & Piercey-Normore, M. D. (2009) Genetically divergent algae shape an epiphytic lichen community on Jack Pine in Manitoba. *Lichenologist* **41:** 69–80.

Ettl, H. & Gärtner, G. (1995) *Syllabus der Boden-, Luft- und Flechtenalgen*. Stuttgart: Gustav Fischer Verlag.

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

Friedl, T. & Gärtner, G. (1989) *Trebouxia* (*Pleurastrales*, Chlorophyta) as a phycobiont in the lichen genus *Diploschistes*. *Archiv für Protistenkunde* **135:** 147–158.

Gasulla, F., Guéra, A. & Barreno, E. (2010) A simple micromethod for isolating lichen phycobionts. *Symbiosis* **51:** 175–179.

Gimeno Molina, B., Barreno, E. & Biosca, E. G. (2016) *Caracterización biotecnológica de bacterias asociadas al liquen* Parmotrema pseudotinctorum. M.Sc. thesis, Universitat de València.

Helms, G. (2003) *Taxonomy and symbiosis in associations of* Physciaceae *and* Trebouxia. Ph.D. thesis, Georg-August Universität Göttingen.

Honegger, R. (1986) Ultrastructural studies in lichens I. Haustorial types and their frequencies in a range of lichens with trebouxioid photobionts. *New Phytologist* **103:** 785–795.

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

Kroken, S. & Taylor, J. W. (2000) Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist* **103:** 645–660.

Leavitt, S. D., Kraichak, E., Nelsen, M. P., Altermann, S., Divakar, P., Alors, D., Esslinger, T. L., Crespo, A. & Lumbsch, T. (2015) Fungal specificity and selectivity for algae play a major role in determining lichen partnerships across diverse ecogeographic regions in the lichen-forming family *Parmeliaceae* (Ascomycota). *Molecular Ecology* **24:** 3779–3797.

Leavitt, S. D., Kraichak, E., Vondrak, J., Nelsen, M. P., Sohrabi, M., Pérez-Ortega, S. St Clair, L. L. & Lumbsch, H. T. (2016) Cryptic diversity and symbiont interactions in rock-posy lichens. *Molecular Phylogenetics and Evolution* **99:** 261–274.

Malavasi, V., Škaloud, P., Rindi, F., Tempesta, S., Paoletti, M. & Pasqualetti, M. (2016) DNA-based taxonomy in ecologically versatile microalgae: a re-evaluation of the species concept within the coccoid green algal genus *Coccomyxa* (*Trebouxiophyceae*, Chlorophyta). *PLoS ONE* **11:** e0151137.

Mansournia, M. R., Wu, B., Matsushita, N. & Hogetsu, T. (2012) Genotypic analysis of the foliose lichen *Parmotrema tinctorum* using microsatellite markers: association of mycobiont and photobiont, and their reproductive modes. *Lichenologist* **44:** 419–440.

Miadlikowska, J., Kauff, F., Högnabba, F., Oliver, J. C., Molnár, K., Fraker, E., Gaya, E., Hafellner, J., Hofstetter, V., Gueidan, C. *et al.* (2014) A multigene phylogenetic synthesis for the class Lecanoromycetes (Ascomycota): 1307 fungi representing 1139 infrageneric taxa, 317 genera and 66 families. *Molecular Phylogenetics and Evolution* **79:** 132–168.

Miller, M. A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November 2010, New Orleans, Louisiana*, pp. 1–8.

Molins, A., García-Breijo, F. J., Reig-Armiñana, J., del Campo, E. M., Casano, L. M. & Barreno, E. (2013) Coexistence of different intrathalline symbiotic algae and bacterial biofilms in the foliose Canarian lichen *Parmotrema pseudotinctorum*. *Vieraea* **41:** 349–370.

Molins, A., Moya, P., García-Breijo, F. J., Reig-Armiñana, J. & Barreno, E. (2018) A multitool approach to assess microalgal diversity in lichens: isolation, Sanger sequencing, HTS and ultrastructural correlations. *Lichenologist* **50:** 123–138.

Moniz, M. B. J, Rindi, F., Novis, P. M., Broady, P. A. & Guiry, M. D. (2012) Molecular phylogeny of Antarctic *Prasiola* (*Prasiolales*, *Trebouxiophyceae*) reveals extensive cryptic diversity. *Journal of Phycology* **48:** 940–955.

Moya, P., Škaloud, P., Chiva, S., García-Breijo, J. F., Reig-Armiñana, J., Vančurová, L. & Barreno, E. (2015) Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems. *International Journal of Systematics and Evolutionary Microbiology* **65:** 1838–1854.

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

Muggia, L., Zellnig, G., Rabensteiner, J. & Grube, M. (2010) Morphological and phylogenetic study of algal partners associated with the lichen-forming fungus *Tephromela atra* from the Mediterranean region. *Symbiosis* **51:** 149–160.

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

Ohmura, Y., Kawachi, M., Kasai, F., Watanabe, M. M. & Takeshita, S. (2006) Genetic combinations of symbionts in a vegetatively reproducing lichen,

Parmotrema tinctorum, based on ITS rDNA sequences. *Bryologist* **109:** 43–59.

Peksa, O. & Škaloud, P. (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (*Trebouxiophyceae*). *Molecular Ecology* **20:** 3936–3948.

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

Piercey-Normore, M. D. & Deduke, C. (2011) Fungal farmers or algal escorts: lichen adaptation from the algal perspective. *Molecular Ecology* **20:** 3708–3710.

Piercey-Normore, M. D. & DePriest, P. T. (2001) Algal switching among lichen symbioses. *American Journal of Botany* **88:** 1490–1498.

Roca-Valiente, B., Divakar, P. K., Ohmura, Y., Hawksworth, D. L. & Crespo, A. (2013) Molecular phylogeny supports the recognition of the two morphospecies *Parmotrema pseudotinctorum* and *P. tinctorum* (*Parmeliaceae*, Ascomycota). *Vieraea* **41:** 333–348.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61:** 539–542.

Ryšánek, D., Holzinger, A. & Škaloud, P. (2016) Influence of substrate and pH on the diversity of aeroterrestrial alga *Klebsormidium* (*Klebsormidiales*, Streptohyta): a potentially important factor for sympatric speciation. *Phycologia* **55:** 347–358.

Sadowska-Deś, A. D., Dal Grande, F., Lumbsch, H. T., Beck, A., Otte, J., Hur, J.-S., Kim, J. A. & Schmitt, I. (2014) Integrating coalescent and phylogenetic approaches to delimit species in the lichen photobiont *Trebouxia*. *Molecular Phylogenetics and Evolution* **76:** 202–210.

Schmull, M., Miadlikowska, J., Pelzer, M., Stocker-Wörgötter, E., Hofstetter, V., Fraker, E., Hodkinson, B. P., Reeb, V., Kukwa, M., Lumbsch, H. T. & Kauff, F. (2011) Phylogenetic affiliations of members of the heterogeneous lichen-forming fungi of the genus *Lecidea sensu* Zahlbruckner (Lecanoromycetes, Ascomycota). *Mycologia* **103:** 983–1003.

Škaloud, P. & Rindi, F. (2013) Ecological differentiation of cryptic species within an asexual protist morphospecies: a case study of filamentous green alga *Klebsormidium* (Streptophyta). *Journal of Eukaryotic Microbiology* **60:** 350–362.

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

Vančurová, L., Peksa, O., Němcová, Y. & Škaloud, P. (2015) *Vulcanochloris* (*Trebouxiales*, *Trebouxiophyceae*), a new genus of lichen photobiont from La Palma, Canary Islands, Spain. *Phytotaxa* **219:** 118–132.

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

Yahr, R., Vilgalys, R. & Depriest, P. T. (2004) Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. *Molecular Ecology* **13:** 3367–3378.

Zwickl, D. J. (2006) *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. Ph.D. thesis, University of Texas at Austin.