



Lichen phycobiomes as source of biodiversity for microalgae of the *Stichococcus*-like genera

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Abstract

The term phycobiome was recently introduced to designate all the microalgae (primary or non-primary) associated with lichen symbioses. Abundant non-primary symbiotic microalgae are usually obtained from lichen isolations, confirming that thalli are a source of biodiversity and new species. In this study, microalgae were isolated from thalli of *Buellia zoharyi*, *Ramalina farinacea* and *Parmotrema pseudotinctorum* collected in the Iberian Peninsula and the Canary Islands. Excluding *Trebouxia* phycobionts, 17 strains similar to *Stichococcus* (*Prasiola* clade) were obtained. Molecular identification was carried out by nuclear ITS sequencing, and a phylogenetic tree was generated from these sequences, and grouping them into 4 clades: *Diplosphaera chodatti*, *Diplosphaera* sp.1, *Deuterostichococcus* sp.1, and *Tritostichococcus coniocybes*. It is also noteworthy that *Diplosphaera* sp.1 was detected and isolated from three phylogenetically distant lichenized fungi (*B. zoharyi*, *R. farinacea* and *P. pseudotinctorum*), which were sampled in ecologically different localities, namely Tenerife, La Gomera and Castellón. These results reinforce the idea of the constant presence of certain microalgae associated with the lichen thalli which, despite not being the main primary photobiont, probably form part of the lichen's phycobiomes.

Keywords Isolation · Phylogeny · *Prasiola* clade Trebouxiphyceae

Introduction

Lichens are iconic examples of symbiotic interactions originated by the cohabitation of heterotrophic ascomycetous and basidiomycetous fungi, e.g., the mycobionts and microalgae (phycobionts) and/or photosynthetic cyanobacteria; in general terms photobionts. Besides the two major lichen symbionts that shape the unique holobiome (symbiogenetic phenotype) of the thallus, an indeterminate number of other microscopic organisms co-occur, intermingled with these associations (Hawksworth and Grube 2020). Due to

the great diversity of organisms integrated in lichen symbiosis (considered as microecosystems) and the need to encompass (or group) them, lichenologists currently use the following terms: holobiome, to refer to the total set of symbiotic or associated organisms located in a lichen thallus (Hawksworth and Grube 2020); microbiome, to refer to all the microorganisms associated with lichens (Sierra et al. 2020); mycobiome, to refer to fungi (not only mycobiont) associated with the lichen thallus (Fernández-Mendoza et al. 2017); and phycobiome, introduced more recently, which is used to designate all the microalgae (primary or non-primary) associated with lichen symbioses (Chiva et al. 2021).

The Mediterranean basin (including Macaronesia) has traditionally been considered as a hotspot of biodiversity (Médail and Quézel 1999); for this reason, a huge number of studies have focused on species growing in this area (Cuttelod et al. 2009). Recently, the phycobiome of numerous lichens has been screened by Next-Generation Sequencing (NGS) (Faluaburu et al. 2019; Xu et al. 2022). Phycobiome diversity has also been analysed by NGS in the lichen species included in this study: *Ramalina farinacea* (L.) Ach. (Moya et al. 2017; Molins et al. 2021) and *Buellia zoharyi* Galun (Molins et al. 2020; Moya et al. 2021). Microalgal diversity

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in the lichen *Parmotrema pseudotinctorum* (Abbayes) Hale, also studied here, was analysed by cloning techniques and Sanger sequencing (Molins et al. 2013; Škaloud et al. 2018). These studies revealed the presence of different *Trebouxia* spp. as the main photobionts, and a different set of minor, or associated microalgal partners.

Trebouxiophyceae is a wide class of algae comprising of coccoid and elliptic unicells, filaments, blades and colony-forming species which occur in diverse terrestrial and aquatic environments. These algal symbionts include the most frequent photobionts in lichen symbioses (Nelsen et al. 2020). Among them, *Trebouxia* Puymaly and *Asterochloris* Tschermak-Woess (Trebouxiiales, Trebouxiaceae) are the most represented genera, but some other taxa, such as *Prasiola* clade microalgae (Prasiolales, Prasiolaceae), have also been found as phycobionts (Sanders and Masumoto 2021).

Stichococcus Nägeli is a microalgal genus of the class Trebouxiophyceae belonging to the *Prasiola*-clade that has recently been split into seven independent lineages (*Desmococcus* F. Brand, *Diplosphaera* Bialosuknia, *Stichococcus*, *Deuterostichococcus* Pröschold et Darienko, *Protostichococcus* Pröschold et Darienko, *Tetrastichococcus* Pröschold et Darienko and *Tritostichococcus* Pröschold et Darienko) which, along with *Pseudostichococcus* L. Moewus, comprise the *Stichococcus*-like group (Pröschold and Darienko 2020). The microalgae of this genera, related to *Stichococcus*, are widely distributed in freshwaters, as well as marine and terrestrial ecosystems (Karsten et al. 2005; Rindi et al. 2007), and they can also be found in harsh polar environments (De Wever et al. 2009; Vishnivetskaya 2009; Khan et al. 2011). Recently, Van et al. (2021) revised the phylogenetic position of *Stichococcus*-like strains, applying chemotaxonomic markers and demonstrating that the patterns of osmolyte production can support green algal taxonomic identifications. Symbiosis is a lifestyle common to many species of *Stichococcus*-like organisms, especially in the lichen family Verrucariaceae (Thüs et al. 2011). The photobionts of the lichen genus *Dermatocarpon* Eschw. have been extensively studied in North America and Europe, where these lichens are associated with green microalgae of the genus *Diplosphaera* (Fontaine et al. 2012, 2013). Moreover, this genus has also been reported as a symbiont partner in the lichen genera *Staurothele* Norman and *Endocarpon* Hedw. (Thüs et al. 2011; Fontaine et al. 2012, 2013). Recently, in the lichen family Agyriaceae, the alga *Deuterostichococcus allas* (Reisigl) Pröschold et Darienko has been identified as a symbiont of the lichen *Placopsis* (Nyl.) Linds. in Antarctic populations (Beck et al. 2019).

In the current study, *Stichococcus*-like microalgae were found to be associated with and were isolated from the lichen species *Buellia zoharyi*, *Ramalina farinacea* and *Parmotrema pseudotinctorum*. To clarify their phylogenetic

position within the *Stichococcus*-like genera clade, the nuclear-coding ITS rDNA of the selected strains were sequenced and phylogenetic analyses were performed. Furthermore, the isolation of these associated microorganisms raised questions about the diversity, ecology and function of the non-primary associated microalgae of lichen phycobionts.

Materials and methods

Lichen material

A total of five specimens were included in this study (Fig. 1; Table 1); three thalli of *Buellia zoharyi* (MT27, TE1 and TE3) were collected; MT27 in Titulcia (Madrid, Spain, 40°07'35"N, 03°33'07"W) growing on Miocene gypsum biocrusts, and TE1 and TE3 from Iguete de San Andrés (Tenerife, Spain, 28°31'44"N, 16°08'49"W) on volcanic substrates, specifically on intermediate lava flows and mafic phonolites. *Ramalina farinacea* (RF5) was collected in a pine forest in El Toro (Castellón, Spain, 39°57'32"N, 00°46'35"W), and a thallus of *Parmotrema pseudotinctorum* (PP2) from San Sebastián (La Gomera, Spain, 28°05'04"N, 17°07'13"W) on ancient basaltic rocks. After collection, the samples were dried at room temperature for one day and stored at -20 °C until processing.

Algal isolation, culture conditions and light microscopy

The lichen thalli were rehydrated and acclimated in a growth chamber two days before carrying out the following procedure. The lichen material was cleaned using a sterile blade to remove visible surface contamination, and it was then superficially sterilized following the Arnold et al. (2009) method. Two different protocols were used to isolate microalgae from these lichen fragments: a) the micro method described by Gasulla et al. (2010) was used to isolate microalgae from the thalli of *R. farinacea* and *P. pseudotinctorum*, and the resulting algal suspension was spread using the triple streak method on sterile 1.5% agar Bold's Basal Media Petri dishes (BBM) (Bold 1949; Bischoff and Bold 1963); and b), the method described in Chiva et al. (2021) was used on *B. zoharyi* thalli, which involved capturing tiny clumps of the algal layer using a Ø 0.3 mm sterile needle and inoculating them directly onto BBM. The isolated microalgae were maintained under a 50 µmol/m²s – 1 photosynthetic photon flux density (PPFD) with a 12 h photoperiod at 18 °C. Well-developed colonies were transferred into a BBM liquid medium using sterile tips under the same conditions of light and temperature.

Fig. 1 Lichen species included in this study. A, *Buellia zoharyi* Galun growing on gypsum soil; B, *Ramalina farinacea* (L.) Ach. in a pine forest; C, *Parmotrema pseudotinctorum* (Abbeyes) Hale on ancient basaltic rocks



Microalgae observations by Differential Interference Contrast (DIC) microscopy were developed using a Nikon Eclipse E-800 microscope with a Nikon DS-Ri1 camera (ICBIBE microscopy service).

DNA extraction, PCR amplification and sequencing

Total genomic DNA from microalgae subcultures was isolated and purified using the DNeasy Plant Mini kit (Qiagen, Hilden, 121 Germany) following the manufacturer's instructions. PCR amplification was performed in 25 µl using EmeraldAmp GT PCR Master Mix (Takara, Shiga, Japan), which required the addition of the template DNA, specific primers and water. The internal transcribed spacer (ITS) region of the nuclear rDNA, which includes ITS1, 5.8 S rDNA and ITS2, was amplified using the primer pair nr-SSU-1780 (Piercey-Normore and DePriest 2001) and

ITS4T (Kroken and Taylor 2000). PCR reactions for ITS loci were performed following Chiva et al. (2021). PCR products were visualized on 2% agarose gels and purified using the Gel Band Purification Kit (GE Healthcare Life Science, Buckinghamshire, UK). 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).

Phylogenetic analysis of the sequences obtained by Sanger sequencing

The identity of the obtained ITS sequences was confirmed by BLAST searches (Altschul et al. 1990) against the GenBank database. In addition, for phylogenetic comparison we downloaded their closest relatives to create a database. The ITS phylogenetic tree was performed with the sequences of

Table 1 Isolates used in the present study, localities sampled, GenBank accession numbers for their ITS sequences, and lichen thalli sources

Lichen sp. (sample)	Locality	Isolation	Isolated microalgae	Phylogeny Code / ITS GenBank accession N.
<i>Buellia zoharyi</i> (MT27)	Titulcia, Madrid, Spain	MT27	<i>Deuteros-tichococcus</i> sp. 1	MT27.1 / OK636223 MT27.2 / OK636228 MT27.3 / OK636227 MT27.4 / OK636226 MT27.5 / OK636225 MT27.6 / OK636224
<i>Buellia zoharyi</i> (TE1)	Iguete de San Andrés, Tenerife, Spain	TE1	<i>Diplosphaera</i> sp. 1	TE1.D / OK636219
<i>Buellia zoharyi</i> (TE3)	Iguete de San Andrés, Tenerife, Spain	TE3	<i>Diplosphaera chodatti</i>	TE3.C / OK636222
<i>Ramalina farinacea</i> (RF5)	El Toro, Castellón, Spain	RF5A	<i>Tritos-tichococcus coniocybes</i>	RF5A.1 / OK636233 RF5A.2 / OK636230 RF5A.3 / OK636229 RF5A.4 / OK636231 RF5A.5 / OK636232
		RF5B	<i>Diplosphaera</i> sp. 1	RF5B.2 / OK636220 RF5B.3 / OK636221
<i>Parmotrema pseudotincturum</i> (PPS2)	San Sebastián, La Gomera, Spain	PPS2	<i>Diplosphaera</i> sp. 1	PPS2.B / OK636234 PPS2.D / OK636235

the newly obtained strains, and with the selected sequences of *Stichococcus*-like organisms from the GenBank. The dataset was aligned using MAFFT v.7.402 software (Katoh and Standley 2013), and then manually modified using MEGA7 (Kumar et al. 2016). The most appropriate nucleotide substitution model (GTR+G) was chosen under the corrected Akaike information criterion using JModelTest v.2.1.6 (Darriba et al. 2012). The phylogenetic tree was inferred using Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. The ML analysis was implemented in RAxML v.8.2.12 using the GTRGAMMA1 substitution model with 1,000 bootstrap pseudoreplicates (Stamatakis

2014). BI was performed in MRBAYES v.3.2.7a (Ronquist et al. 2012). There were two parallel Markov Chain Monte Carlo (B/MCMC) running with six chains simultaneously, each initiated with a random tree for 10 million generations, and the trees were sampled every 100 generations for a total sample of 20,000 trees. Phylogenetic trees were visualized in FIGTREE v 1.4.3 (Rambaut 2017). All the analyses were run in the CIPRES Science Gateway v 3.3 web portal (Miller 2012).

Results

Isolation of unialgal strains

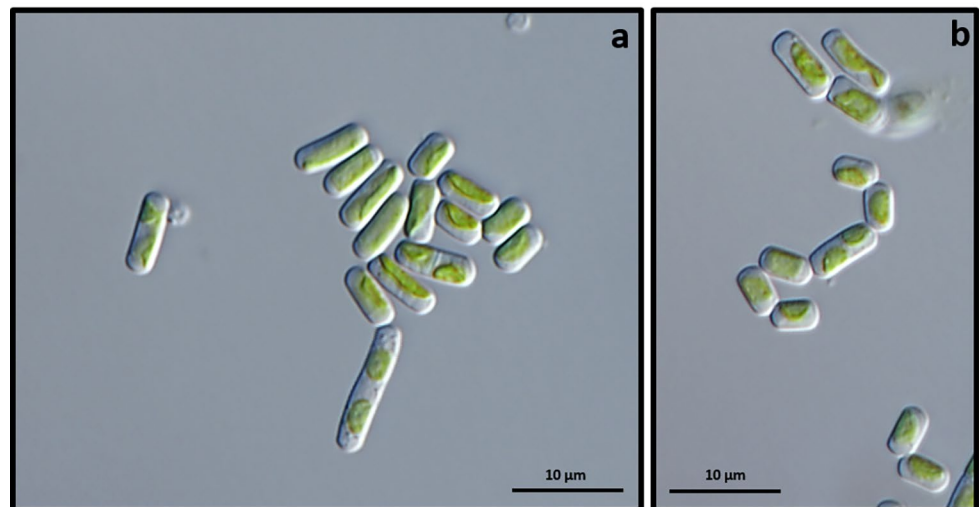
Trebouxia microalgae were obtained as the primary symbiont in the five lichens thalli included in this study: MT27 (Moya et al. 2021), TE1 (Molins et al. 2020), TE3 (Molins et al. 2020), RF5 and PPS2 (unpublished); however, they are not the main interest of this study. Other microalgae strains were also obtained from the phycobiome (Table 1). Six strains (MT27.1, MT27.2, MT27.3, MT27.4, MT27.5 and MT27) were isolated from *B. zoharyi* thalli (MT27). TE1.D and TE3.C strains were obtained from *B. zoharyi* TE1 and TE3, respectively. Seven strains (RF5A.1, RF5A.2, RF5A.3, RF5A.4, RF5A.5, RF5B.2 and RF5B.3) were isolated from *R. farinacea* thalli (RF5), and two strains (PPS2.B and PPS2.D) were isolated from *P. pseudotincturum* thalli (PPS2).

Due to complications related to contamination by other organisms, only the TE1.D strain could be successfully recultured. Therefore, axenically unialgal cultures were achieved only with this strain, which was subsequently deposited in the ASUV Collection of symbiotic microalgae at the University of Valencia under code ASUV135 (www.asuvalgae.com). In Fig. 2, the microalga *Diplosphaera* sp. ASUV135 can be observed by DIC. This strain, like all other *Stichococcus*-like organisms, is characterized by its simple morphology with cylindrical, or short cylindrical cells, containing plate-shaped chloroplasts without pyrenoids (Fig. 2a). The cells sometimes form short filaments (Fig. 2b).

Phylogenetic analyses

The resulting alignment included 64 taxa for ITS analyses (854 characters), 17 newly obtained in this study, and the remaining downloaded from the GenBank. BI and ML phylogenetic hypotheses were topologically congruent. The inferred tree of *Stichococcus*-like organisms was also topologically congruent with those of previous studies (Hodač et al. 2016; Pröschold and Darienko 2020) and grouped the

Fig. 2 Morphology of *Diplosphaera* sp. ASUV135 (TE1.D). (a): Cylindrical cell characteristics of *Stichococcus*-like organisms, (b): Cells forming short filament



newly sequences obtained as four species-level lineages (Fig. 3; our sequences are highlighted in bold). Five microalgae isolated from *R. farinacea* (RF5A.1, RF5A.2, RF5A.3, RF5A.4 and RF5A.5), clustered together with sequences from the genus *Tritostichococcus*, specifically matched with *T. coniocybes* (Letellier) Pröschold et Darienko (Fig. 3), with a highly supported clade (ML/Bi: 100/100). Strains isolated from the *B. zoharyi* thallus (MF27) were grouped together with *Deuterostichococcus* sequences in a highly supported independent clade (ML/Bi: 99/100) as a new species-level lineage: named *Deuterostichococcus* sp. 1 (Fig. 3) close to *D. deasonii* (Neustupa, Elias et Sejnová) Pröschold et Darienko. Microalgae isolated from the remaining *B. zoharyi* (TE1: TE1.D and TE3: TE3.D), *R. farinacea* (RF5B.2 and RF5B.3) and *P. pseudotinctorum* (PPS2: PPS2.B and PPS2.D) thalli, clustered with several *Diplosphaera* genus sequences. Specifically, TE3.D was grouped with *D. chodatii*, and TE1, RF5B and PPS2 formed a new species-level lineage (ML/Bi: 100/100), named *Diplosphaera* sp. 1 (Fig. 3).

Discussion

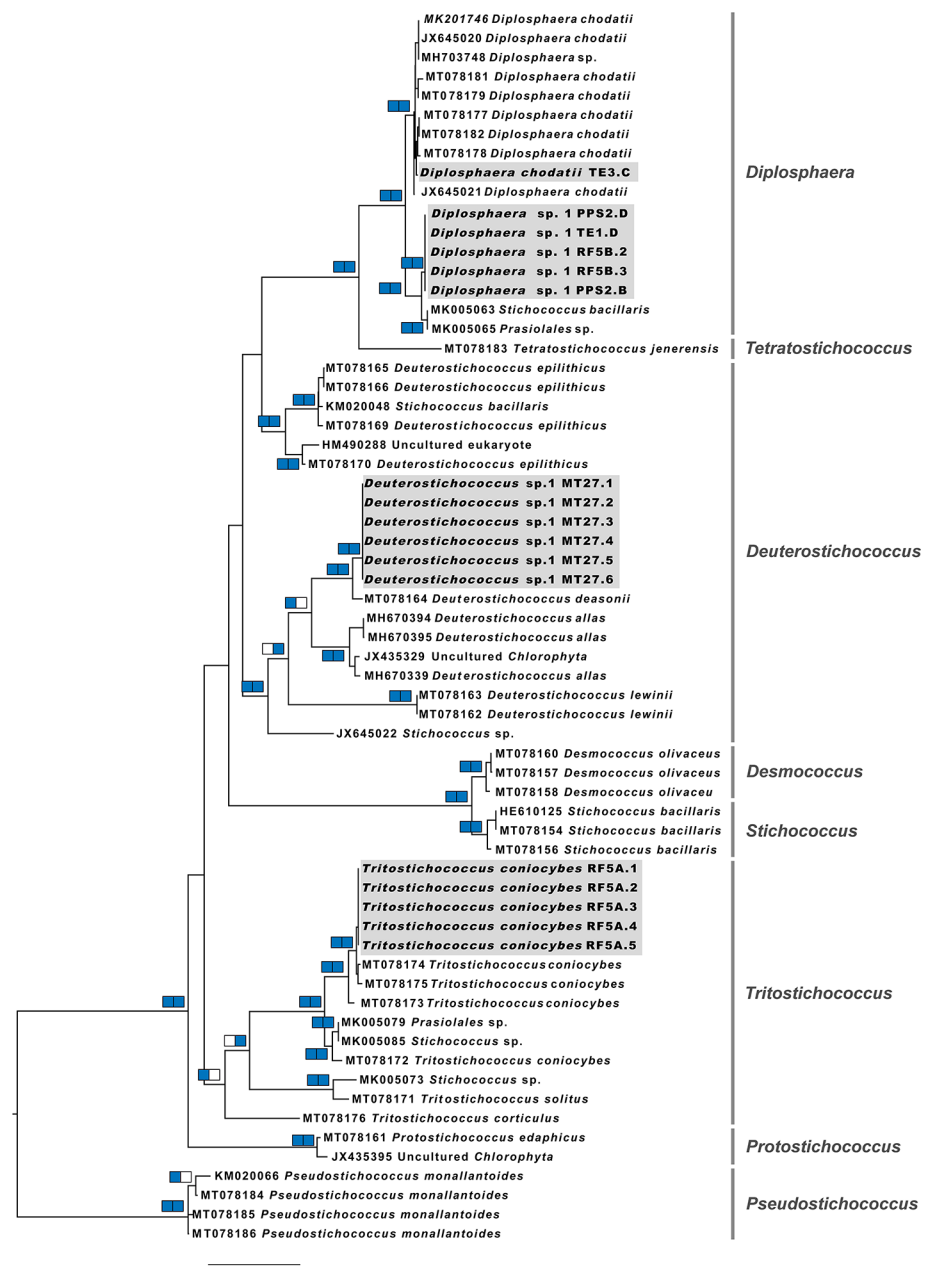
Stichococcus-like organisms have frequently been detected as free-living microalgae in different types of habitats (Pröschold and Darienko 2020); numerous studies detected *Stichococcus*-like organisms as the symbiont (Sanders and Masumoto 2021 and the references therein). In the lichens used for isolations in this study, strains of three different genera, belonging to *Stichococcus*-like microalgae, were obtained associated with the phycobiome: *Diplosphaera*, *Deuterostichococcus* and *Tritostichococcus*. In particular, some lineages of these three genera were previously described as lichen symbionts. In the case of *Diplosphaera* spp. they were detected as the main primary symbiont in

numerous Verrucariaceae genus (Thüs et al. 2011); *Deuterostichococcus allas* and *D. deasonii* in *Placopsis* spp. and *Staurothele clopima* (Wahlenb.) Th. Fr., respectively (Hodač et al. 2016; Beck et al. 2019); and *Tritostichococcus coniocybes* in *Chaenotheca* sp. (Pröschold and Darienko 2020).

In contrast, these *Stichococcus*-like microalgae were also found as a minor fraction of the phycobiomes of other lichen species. Moya et al. (2017) analysed the microalgal diversity in a single thallus of the lichen *R. farinacea* by applying a 454-pyrosequencing approach. This analysis allowed the detection of 31 OTUs representative of different genera of microalgae, including among them (as a minor partner) the genus *Diplosphaera*. Similar results were found for *B. zoharyi* thalli from the Iberian Peninsula and the Balearic Islands using Illumina, where microalgae of the genus *Diplosphaera* and *Pseudostichococcus* were detected as a minor fraction in all the localities (Moya et al. 2021). These minor partners are commonly detected in lichen studies when NGS is applied. Although these microalgae seem not to play a major role in lichen symbioses, they may likely participate in the composition of phycobiomes because of the frequency with which they are detected.

A total of 17 microalgae strains were isolated from the five lichen thalli used in this study. Specifically, two distinct lineages were isolated from *R. farinacea*: *Diplosphaera* sp. 1 and *T. coniocybes*. Three lineages were isolated from *B. zoharyi*: *D. chodatii*, *Diplosphaera* sp. 1 and *Deuterostichococcus* sp.1. In the case of *P. pseudotinctorum*, the *Diplosphaera* sp. 1 lineage was also isolated. Therefore, it is also noteworthy that *Diplosphaera* sp.1 was detected and isolated from three different phylogenetically distant lichen species (TE1: *B. zoharyi*, RF5B: *R. farinacea* and PPS2: *P. pseudotinctorum*) which were sampled in ecologically different localities, namely Tenerife, La Gomera and Castellón. These results reinforce the idea of the constant presence

Fig. 3 Molecular phylogeny based on ITS sequences of 64 taxa of *Stichococcus*-like organisms. The blue squares represent significant statistical clade support obtained with PhyML (left square, bootstrapping probabilities $\geq 70\%$) and MrBayes (right square, posterior probabilities ≥ 0.95). Sequences obtained in this study are highlighted in bold and grey



of certain microalgae associated with the phycobiontes of lichen thalli, in spite of the fact that they are not the main primary phycobiont. In addition, recent studies using NGS techniques with several genera of lichens have detected different taxa of microalgae associated with the phycobiontes and represented as minor fractions. Some of them occur repeatedly, e.g., *Stichococcus*, *Coccomyxa* or *Elliptochloris* (Muggia et al. 2013; Noh et al. 2020; Vančurova et al. 2020). The repetitive occurrence of these taxa suggests that these microalgae may be constantly associated with the lichen symbiosis. However, discussing these results in depth is complicated by the fact that these studies do not focus on these less frequent taxa and often only mention them at the

genus level, without going deeper at the species level. Furthermore, the availability of these dataset is limited, as they cannot be directly analysed by BLAST searches as they are released as bioprojects.

The primary symbiotic microalgae in the lichen species used in this study have been extensively studied. *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner and *Trebouxia* sp. TR9 have been detected as the primary microalgae in studies on *R. farinacea* (del Campo et al. 2010; Moya et al. 2017; Molins et al. 2021). Similarly, the lichen *B. zoharyi* (Chiva et al. 2019), was shown to be the primary phycobiont in a set of four *Trebouxia* spp. (Molins et al. 2020; Moya et al. 2021). Microalgae of *P. pseudotinctorum* from

the Canary Islands were also analysed (Molins et al. 2013, Škaloud et al. 2018), and revealed three *Trebouxia* spp. as the primary symbionts. Therefore, diverse microalgae of the genus *Trebouxia* were described as the symbiotic primary phycobionts in all the lichen thalli used in this study. For this reason, the microalgae detected as lichen phycobiome players in the lichens analysed here, appear to be minor constituents with an, as yet, unknown role.

As reported by Bordenave et al. (2022), the success rate of photobiont isolation from lichens is relatively low. This is mainly due to the difficulty of obtaining pure microalgae strains, as they present a high risk of contamination by fast-growing organisms in the culture medium used, as was the case for most of the strains isolated in this study. Despite this, abundant non-primary microalgae symbionts are usually obtained in lichen isolations, confirming the thallus as a source of biodiversity and new species (Chiva et al. 2021). The location and origin of these microorganism could be questioned, hence surface sterilization of the target lichen fragment is essential in the isolation procedure. However, total sterilization of the surface cannot be guaranteed in lichens which are very close to the substrate. Surface cleaning is common both in studies related to the isolation and description of new species (e.g., *Watanabea lichenicola* Chiva, Dumitru, Bordenave & Barreno in Chiva et al. 2021) and in diversity and metabarcoding analyses in which symbiosis-associated microorganisms are the target, and assume the surface sterilization process as key to ensure that the results obtained come exclusively from organisms located inside the lichen thalli (Arnold et al. 2009; Muggia et al. 2013). In addition to surface sterilization, with the isolation protocol of Chiva et al. (2021) algae are captured from areas of the algal layer that have been removed with a sterile scalpel from the upper cortex. This ensures that the colonies growing in the culture dish come from within the thallus.

In this case, *Stichococcus*-like organisms remain mainly hidden behind the primary *Trebouxia* and, under the above premises, these strains should be considered as members of the lichen phycobiomes in the lichens analysed here. These non-primary microalgae detected may have a symbiotic capacity, as described in other studies with related microalgae species (Sanders and Masumoto 2021 and references therein). In fact, species of the three genera of microalgae isolated in this study are the principal algal partner in some lichen genera. Hence, we cannot rule out their possible photosynthetic role, acting as a non-primary algae in the symbiosis.

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Daniel Sheerin revised the English manuscript.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Salvador Chiva. The first draft of the manuscript was written by Salvador Chiva and all the authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript.

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Ethical statement

Conflict of interest The authors declare that they have no competing interests.

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