Symbiosis

Multidisciplinary approach to describe Trebouxia diversity within lichenized fungi Buellia zoharyi from the Canary Islands

--Manuscript Draft--

Manuscript Number:	SYMB-D-20-00159R1
Full Title:	Multidisciplinary approach to describe Trebouxia diversity within lichenized fungi Buellia zoharyi from the Canary Islands
Article Type:	Festschrift Barreno
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Funding Information:	Ministerio de Economia y Competitividad (CGL2016-79158-P) — Dr. Patricia Moya
	Generalitat Valenciana (PROMETEOII/2013/021) — Dr. Patricia Moya
	Generalitat Valenciana (PROMETEO/2017/039) — Dr. Patricia Moya
Response to Reviewers:	Ref: Ms. No. SYMB-D-20-00159 Title: Temperature as a bioclimatic factor affecting association patterns in Buellia zoharyi from the Canary Islands Note for the Editor: Dear David there was a mistake related to the author´s order in the submission menu, the correct order is the one that appear in the doc. Sorry for this inconvenience Authors: Arantzazu Molins1, Salvador Chiva1, Ángeles Calatayud2, Francisco Marco3, Francisco García-Breijo4,5, José Reig-Armiñana1,4, Pedro Carrasco3, Patricia Moya1 Editor comments Please consider the comments and recommendations for revision by the Reviewers. The recommendations for changes, though in some cases substantial, are well worth making as I am sure the paper will be much improved as a consequence. If there are any recommendations you do not wish to comply with, please identify these and state

your reasons in an E-mail with the attached electronic version of your revised manuscript.
Thank you very much for your recommendations. Substantial changes have been made in the manuscript. See def version and with CC attached

Reviewer Comments:

Reviewer 1

Line 93: check ever – present
Done. See line 92
Line 120: occur instead of occurs
Done. See line 116
Line 253 algal strain instead of algae strain
Done. See line 277

Reviewer 2

I would suggest that this be published without the additional physiological measurements. These need to be much more intensively and carefully studied before we can know if the observed patterns have anything to do with the ability of the strains to display differences in acclimation and adaptation to temperature.
Thank you very much for your recommendations. We have reorganized, reduced and re-written the complete manuscript. We decided not to exclude the physiological measurements but we have changed the approach of this part of the study, because as the referee mentioned additional measurement should be included to confirm the influence of the temperature in the photobiont adaptation. However, we consider that each Buellia zoharyi photobionts showed different Chlorophyll a fluorescence responses to different temperatures (20º C and 17º C) as according to the Fv/Fm measurements (Fig. 5a). As shown in this Fig. Trebouxia sp. `arnoldoi´ and T. cretacea were significantly affected by temperature, with the lowest Fv/Fm values at 17º C. However, Trebouxia asymmetrica showed similar values at 20º C and 17º C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them. Therefore, those differences are described in the manuscript.

I certainly don't believe that the data presented justify the title of the m/s: "Temperature as a bioclimatic factor affecting association patterns in Buellia zoharyi". I personally would doubt if temperature has much influence, but in any event, much more work is needed.
As the referee recommended we have changed the title: Multidisciplinary approach to describe Trebouxia diversity within lichenized fungi Buellia zoharyi from the Canary Islands

Briefly, my feeling is that the differences in temperature between the sites (17 and 20oC) are too small to have selected different photobionts. Studies that have focused on thermal adaptation have typically used populations of lichens growing in areas with much greater differences in temperature. For example, Sahu et al. (2019) measured NPQ etc., in Phaeophyscia hispidula and Flavoparmelia caperata growing along a large altitudinal gradient, with average temperatures between sites varying by more than 20oC. In the present study with Buellia, differences in photobiont composition would seem to be unlikely to be related to such small differences in temperature when other factors e.g. N supply may vary between sites.
As mentioned before, we agree with the referee that additional measurement should be included to confirm the influence of the temperature or other bioclimatic factors in the photobiont adaptation hypothesis.
We know, Sahu et al (2019) studied variations in microclimatic attributes and their effects on photosynthetic efficiency and analyzed four elevational ranging from 1950 to 3508 m. They detected that extreme variations in air temperature (5.75–31. 65º C) and other factors were imperative in controlling species richness, distribution and photosynthetic quenching of lichen flora in the region. However, in this work, Buellia zoharyi populations included in this study grown in areas with similar ecological conditions except for the temperature although other factors could be influence on their performance

Moreover Casano et al (2011), as mentioned in the manuscript (see lines 527-539). To search for physiological differences between the two R. farinacea phycobionts, studied the effects of temperature and light on the growth and photosynthetic traits of isolated and cultured T. jamesii and Trebouxia sp. TR9 algae. They showed the fresh weight reached by both microalgae after 30 days at 17°C or 20°C and 15–100 μmolm−2s−1 of PAR. Both species grew better at the lower temperature; however, at 20°C the negative impact of the high temperature was less on TR9 than on TR1, whose growth was almost inhibited. In fact, since the low algal biomass at 20°C significantly increased the experimental error of fluorescence measurements, rendering non-reliable data, so the experiments in Casano et al (2011) were carried out only in cultures growing at 17°C. Therefore, the influence of only 3-4 ºC of temperature in the Trebouxia spp. was previoulsy reported by the mentioned authors.
On the contrary, we did not find such differences in the growth temperature, and thus, we performed the experiments at both 17º C (mean annual temperature in Fuerteventura and Lanzarote) and 20º C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures (20º C and 17º C).
In the same way, I cannot accept that experiments that quantified the responses of the photobionts to the two temperatures can explain the observed differences in photobiont composition. Thus, while it is interesting that strains cultured at 17oC compared with 20oC display slightly lower Fv/Fm values, it is hard to really interpret this observation without more data. It certainly does not correlate simply with NPQ values.
See previous answer
From the data in Figure 5, it appears that algae cultured at 17oc have slightly higher ETR rates, which is not consistent with the Fv/Fm data. In general, I found the logic in the paragraphs discussing the fluorescence data muddled and difficult to follow, and furthermore, does not refer back to the figures. But ultimately, based on the data presented, photobiont composition cannot be related to the response of the photobionts to temperature.
We have rewritten this part of the document to clarify it
I am rather skeptical that individual spot measurements of phytohormone levels will tell us much about the stress tolerance / differences in the different photobionts. The work of Pichler et al. (2020) cited by the authors illustrates one possible approach. Here, first secretion of hormones was studied, and second changes in the levels of the hormones induced by light and desiccation. In theory, a study comparable to this with the different strains / geographical isolates could be valuable, but the data presented are not helpful. In conclusion therefore, my recommendation therefore is that the physiology sections are deleted and the m/s be re-submitted without them.

Thank you very much for your recommendations. Due to the scarce information related to the phytohormone content in microalgae available in the literature, we agree with the referee that a study comparable to Pichler et al. (2020) with different strains / geographical isolates exposed to several growth conditions (control and stress), would be valuable to understand photobiont adaptation, given the well know relationship between phytohormones and adaptation to environmental conditions. However, in the present study this analysis was included as a preliminary technique in order to complement the photobiont characterization. In this sense, we have been able to detect different phytohormone profiles for the three isolated photobionts, an observation that could suggest different signaling needs for each of them. We consider this data an interesting start point for further studies that could help to determine how phytohormone signaling could contribute to explain the local adapted photobiont hypothesis. In order to clarify our point of view to a future reader, we have reordered the information regarding phytohormone data in material and methods, results and discussion in the manuscript.

Minor Comments
In generalreplace phycobiont with photobiont
Done. See new manuscript
Line 99Meaning of "outcompete" not clear
We have changed adapt and outcompete for thrive. See line 98
Line 123Meaning of "communities" not clear - "populations"?
Done. See line 119
Line 126something is missing before "population"
Done. See line 122

	Line 127"have" rather than "showed" Rephrased. See lines 123-125 Line 128a "biocrust" is not an ecological condition Rephrased. See line 125 Line 139"document" rather than "depict" We have modified depict for describe. See line 136 Line 144"They were characterized by means of molecular, microscopy and spectrometry." Please turn into a grammatical sentence. Done. See line 141 Line 291"grau"? Grade. See line 247 Line 306replace "with" with "with the" Done. See line 262 Line 402First paragraph of the Discussion should summarize the findings of the study, not the aims!! Thank you for the suggestion. We have rewritten these paragraph. See lines 406-409 Line 420better give area in square km Sentence rewritten. See lines 418-419 Line 486"resolutive", odd word, please re-phrase Sentence deleted. Line 538"heterogeneities of Chl fluorescence" - is this data really used? If not, maybe delete reference to it. We agree with the referee. Thank you for the recommendation. We have deleted this paragraph and reference Line 554 "which could indicate sensitivity to photoinhibition" - rather an odd statement, given that the photobionts were cultured at 50 umol m-2 s-1? Sentence deleted Line 937Caption to Figure 5. "Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas." This text seems to be in the wrong place? Paragraph included in material and methods. See lines 299-303

Journal: Symbiosis

Article type: Special Issue Festschrift Barreno

Title: Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia zoharyi* from the Canary Islands

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Running title: *Trebouxia* species within *Buellia zoharyi* from the Canary Islands

Abstract

The Canary Islands are famous for their extraordinary biodiversity; however, lichenized algae have only been studied partially. *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species that usually occurs in semi-arid areas of the Mediterranean, but occasionally some interesting communities of this species grow on basaltic lava flows in Lanzarote, Fuerteventura and Tenerife. Those three locations showed similar ecological conditions, but different mean annual temperatures. Here we applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi* covering the entire described range of distribution in the Canary Islands. Photobionts were characterized in symbiosis using molecular and microscopic techniques. Different *Trebouxia* spp. were detected as primary photobiont in each island (*Trebouxia cretacea*-Fuerteventura, *T. asymmetrica*-Lanzarote and *Trebouxia* sp. *`arnoldoi´*-Tenerife). Coexistence of various *Trebouxia* spp. within a thallus were detected by using specific primers-PCR. Those three photobionts were isolated and cultured under laboratory conditions. Different phytohormone profiles were obtained in the isolated strains which suggest different internal signalling needs. In addition, we characterized the response of the isolated strains to different temperatures using chlorophyll fluorescence. *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In contrast, *Trebouxia* sp. *`arnoldoi´* and *T. cretacea* were more sensitive to changes in growing temperature decreasing Fv/Fm at 17º C. Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the region, and suggest that bioclimatic factors could influence the myco/photobiont association patterns.

Keywords: coexistence, isolation, microalgae, photosynthesis, symbiosis, ultrastructure

Declarations

Funding Funding for field and laboratory work for this study was provided by the Ministerio de Economia y Competitividad (MINECO and FEDER, Spain) (CGL2016-79158-P) and Prometeo Excellence in Research Program (Generalitat Valenciana, Spain) (PROMETEOII/2013/021; PROMETEO/2017/039). Daniel Sheerin revised the English manuscript.

Competing interests The authors declare no competing interests

Availability of data and material GenBank

Code availability LSU rDNA: MT458607-MT458609; nrITS MT458610-MT458618

Authors' contributions PM, AM and SC conceived the study and designed the laboratory part of the study. SC, PM and AM carried out laboratory work. FG-B and JR-A analyzed microscopic images. AC and PM performed photosynthesis measurements. FM and PC performed phytohormone quantifications. AM and PM analyzed all data and wrote the manuscript. All authors edited and approved the final version of the manuscript.

Acknowledgments

Dr. Arnoldo Santos was involved in the design of the surveys and the sample collection, also in the ecology and bioclimatic information of the locations. Daniel Sheerin revised the English manuscript. We dedicate this article to Eva Barreno in honour of her retirement.

Introduction

The Canarian Archipelago contains eight volcanically active islands proximal to the African continent and the High Atlas Mountains. The origin and magmatic evolution of these islands have been contentious issues for several decades and make this particular archipelago unique for biodiversity studies. The Canary Islands are famous for their extraordinary diversity in lichenized and lichenicolous fungi (Van den Boom and Etayo 2006; Hernández Padrón and Pérez Vargas 2010). The diversity of lichenized algae has only been studied partially for *Tephromela atra* (Muggia et al. 2010), *Ramalina farinacea* (Casano et al. 2011; del Campo et al. 2013; Moya et al. 2017), *Lecanora rupicola* (Blaha et al. 2006), *Parmotrema pseudotinctorum* (Molins et al. 2013; Škaloud et al. 2018), *Stereocaulon vesuvianum* (Vancurová et al. 2015), *Psora decipiens* (Moya et al. 2018) and *Cladonia* spp. (Moya et al. 2015).

Lichen symbioses are currently considered as microecosystems in which multispecies assemblages are hosted in the lichen thallus (holobiont), formed by the two major lichen symbionts, e.g. the mycobiont (fungal partner) and the photobiont (a population of photosynthetic green or blue-green algae). According to the most recent investigations, many other fungi (Muggia and Grube 2018; Smith et al. 2020), non-photosynthetic bacteria (e.g. Aschenbrenner et al. 2014; Grube et al. 2015; Cernava et al. 2017; Sierra et al. 2020) and photosynthetic green microalgae co-inhabit within the lichen thalli, giving rise to the peculiar phenotypes, and which may contribute to the diverse patterns of secondary metabolites (Spribille 2018) and environmental adaptation. In terms of lichen photobionts, intrathalline microalgal diversity, e.g. multiple photobiont species within a single lichen thallus, has previously been observed in a number of lichen symbioses (Dal Grande et al. 2014; Muggia et al. 2013; Moya et al. 2017; Škaloud et al. 2018). In some cases, algae with different physiological performances are present in lichen thalli, potentially facilitating the success

of these lichens in a wide range of habitats and geographic areas and/or in changing environmental conditions (Casano et al. 2011; del Hoyo et al. 2011).

Muggia et al. (2020) highlight the need for an integrative taxonomic approach, incorporating morphological and physiological data from axenic cultures with genetic data, to establish a robust, comprehensive taxonomy for *Trebouxia.* Complementation with ecophysiological studies needs to be employed to explain how lichens thrive in extreme environments (Sadowsky and Ott 2012). Lichen photobionts show a particular photosynthesis performance and have evolved alternative photosynthetic-machinery protective mechanisms in response to their special ecophysiology (Gasulla et al. 2019). The photosynthesis responses to abiotic factors, like temperature and light conditions in axenic cultures of photobionts, reflects the ecophysiological plasticity of this symbiosis as a mechanism, allowing the lichen to adapt to changing and often stressful environments (Casano et al. 2011).

Phytohormones are chemical messengers involved in several physiological, stress response and biochemical processes of higher plants at very low concentrations. Phytohormone composition has been characterized in only a few algae (Yokoya et al. 2010; Gupta et al. 2011; Wang et al. 2014), but recently, Pichler et al. (2020) studied phytohormone composition in aeroterrestrial Trebouxiophyceae, including three lichen- forming microalgae. The results obtained in this study contribute to valuable baseline information for further studies into the roles of phytohormones in microalgae. Including this technique to characterize isolated symbiotic microalgae could help to understand the ecologically adapted photobionts hypothesis.

Buellia zoharyi is a circum-Mediterranean/Macaronesian species forming crustose placodioid lichens that usually occur in biocrusts in semi-arid areas of the Mediterranean region (Gutiérrez-Carretero and Casares-Porcel 2011). Specifically, this species predominantly grows on gypsum soils (Crespo and Barreno 1975; Barreno 1994; Trinkaus and Mayrhofer 2000), but occasionally some interesting populations of this species grow on basaltic lava flows in the Canary Islands (Etayo 2011; Giralt and Van den Boom 2011; Roux and Poumarat 2015). The presence of this lichen species in the Canary Islands was reported in Lanzarote (Trinkaus and Mayrhofer 2000), Fuerteventura (Van den Boom and Etayo 2006) and Tenerife (Chiva et al. 2019), and populations in the other four Islands must be scarce or even non-existent. These three locations have similar ecological conditions e. g. the same high irradiance (5.5 – 5.7 KWh/m2) moreover, in the three locations *B. zorayi* grown forming biocrusts in areas with sparse vascular vegetation. However, the WordlClim database reported different mean annual temperatures (MAT): 17º C in Fuerteventura

and Lanzarote, and 20º C in Tenerife (https://www.worldclim.org; Hijmans et al. 2005). Chiva et al. (2019) analyzed mycobiont diversity in *B. zoharyi* in the Mediterranean region including those three Canary Island locations. They found low genetic diversity and two geographically differentiated haplogroups: one including populations from the Iberian Peninsula to Azerbaijan, and the other from the southern Iberian Peninsula, North Africa and the Canary Islands. Complementary analyses of *B. zoharyi* concerning the range of associated photobionts and their ecophysiological traits will be necessary to identifying historical and ecological factors at the basis of the evolutionary history of this group of soil- dwelling taxa.

Here we applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi* settled in the Canary Islands. Populations included in this study cover the entire described range of distribution in the archipelago. Photobionts were characterized in symbiosis using molecular and microscopic techniques. Coexistence of various *Trebouxia* spp. were detected by using specific primers-PCR. Representative photobionts were isolated and cultured under laboratory conditions. We characterized the isolated strains using molecular, microscopy and spectrometry techniques. Their responses to different temperatures were monitored using chlorophyll fluorescence to answer if this bioclimatic factor could influence the myco/photobiont association patterns.

Material and methods

Sampling and DNA extraction

In this study, 46 thalli of *Buellia zoharyi* collected from Lanzarote, Tenerife and Fuerteventura were analyzed (Table 1S). We included fresh (N=43) and herbarium samples (N=3). Fresh specimens were air-dried for one day after sampling and then stored at -20°C. Lichen thalli were examined under a stereomicroscope to remove soil particles and were immersed sequentially in ethanol and NaOCl (Arnold et al. 2009) to remove surface contaminants and to ensure the intrathalline origin of the sequenced microalgae. Fragments from different parts of each thallus were randomly excised and pooled together. Total genomic DNA was isolated and purified using the DNeasy Plant Mini kit (Qiagen, Hilden, 121 Germany) following the manufacturer's instructions.

Primary photobiont PCR amplification and Sanger sequencing

Two algal loci were amplified; a region of the chloroplast LSU rDNA gene using the algal specific primer pair 23SU1 and 23SU2 (del Campo et al. 2010a) and the nrITS (internal transcribed spacer) using the primer pair nr-SSU-1780 (Piercey-Normore and DePriest 2001) and ITS4 (White et al. 1990). PCR reactions were performed following Moya et al. (2018). The PCR products were

visualized 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 ABI 3730XL using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Sanger sequences were visualized and manually evaluated with Chromas 2.6.6.0 (http://technelysium.com.au/wp/chromas/).

Trebouxia photobiont phylogenetic analysis

The nrITS and LSU rDNA datasets were collapsed using TCS 1.21 (Clement et al. 2002). The dataset included the newly determined nrITS and LSU rDNA sequences from thalli and isolated photobiont (accession numbers LSU rDNA: MT458607-MT458609; nrITS: MT458610-MT458618), and a selection of 30 *Trebouxia* species sequences available from the Culture Collection of Algae at Goettingen University (SAG), from the Culture Collection of Algae at the University of Texas (UTEX) and from the Symbiotic Microalgal Collection of the University of Valencia (ASUV, *Trebouxia* sp. TR9 and *Trebouxia crespoan*a). We included *Asterochloris mediterranea* as an outgroup.

A multiple alignment was built using MAFFT 7.0 (Katoh and Standley 2013) using default parameters. Aligned sequences were improved by eliminating the ambiguous regions using Gblocks 0.91b (Castresana 2000) with the least stringent parameters. This software allows conflicting regions in the alignment to be automatically removed. Both loci were concatenated, yielding an alignment of 1329 characters. The final matrix contained 42 ITS rDNA and 33 LSU rDNA sequences.

For each locus, the most appropriate substitution model was estimated using the Akaike information criterion (AIC) using JModelTest 2.1.4 (Darriba et al. 2012). The most appropriate nucleotide substitution models for nrITS and LSU rDNA were GTR+I+G and GTR+G, respectively. The phylogenetic trees of both loci were inferred by Bayesian inference (BI) and Maximum Likelihood (ML) approaches carried out on partitioned datasets using the different substitution models selected by JModelTest. ML analysis was implemented in RAxML 8 (Stamatakis 2014) using the GTRGAMMA substitution model. Bootstrap support (BS) was calculated based on 1,000 pseudoreplicates (Stamatakis et al. 2008). BI was carried out in MrBAYES 3.2 (Ronquist et al. 2012). Settings included two parallel runs with six chains over 20 million generations, starting with a random tree and sampling after every 200th step. We discarded the first 25% of the data as burn-in, and the corresponding posterior probabilities (PPs) were calculated from the remaining trees. Estimated sampled sized (EES) values above 200, and potential scale reduction factor (PSRF)

values approaching 1,000, were considered indicators of chain convergence. The phylogenetic tree was visualized in FIGTREE 1.4.2 (Rambaut 2014; http://tree.bio.ed.ac.uk/software/figtree/). All analyses were run at the CIPRES Science Gateway 3.3 webportal (Miller et al. 2010).

Microscopic examinations "in thallus".

The ultrastructure of the photobionts was characterized by transmission electron microscopy (TEM) from selected thalli (LA7, LA22, TE1, TE10 and FU1). For TEM, the cells were fixed and dehydrated as described in Molins et al. (2018a). Samples were embedded in Spurr's resin according to the manufacturer's instructions. Sections (90 nm) were cut and mounted as described in Moya et al. (2018). 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.

Secondary *Trebouxia* strains detected by specific PCR primers

To detect the presence of secondary *Trebouxia* species in each thallus, PCR were performed using as templates each PCR from the primary photobiont (obtained with the primer pair nr-SSU-1780 / ITS4) and re-amplifying with specific primer pairs (Table 2S). These specific forward and reverse primers were designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia cretacea* and *Trebouxia* sp. *`arnoldoi´*.

Isolation, PCR identification and propagation of microalgae strains.

Photobionts were isolated from selected thalli (LA7, TE1 and FU1) using two protocols: a) the micromethod described by Gasulla et al. (2010), where the resulting algal suspension was diluted with sterile water and spread using the 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 Muggia et al. (2014) where tiny clumps of the algal layer were inoculated directly into BBM. The isolated algae were maintained under a 50 μmol/ $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) with a 12 h photoperiod at 20°C. Subsequent subcultures were performed until we obtained a unialgal culture and fast PCR microalgae identification was performed directly from the colonies as described in Molins et al. (2018b).

Microscopic investigations of photobionts "in culture".

Light microscopy (LM) and Epifluorescent Microscopy (EFM) was performed on selected unialgal cultures on the 21st day of cultivation at 20º C. All LM and EFM observations were carried out with an Olympus Provis AX 70 fluorescence microscope equipped with an Infinity 2–3 C Lumenera® digital camera and analyzed with "Infinity Analyze" Software. For EFM, an Olympus U-ULS 100 HG epifluorescence system with U-MWBV (excitation filter 400–440 nm, dichroic mirror 455 nm, barrier filter 475 nm) cubes was used.

Phytohormone content determination

Endogenous phytohormone levels of 21-day-old microalgae cultures grown at 20º C were determined according to the protocol of Durgbanshi et al. (2005). Lyophylized microalgae samples were processed by extraction in 5 ml of distilled water after fortifying them with internal standards: [$^2H_6$] - abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)), dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation according to Kristl et al. (2005)), [$^2H_2$]-indole-3-acetic acid (IAA) (10 ng, Sigma-Aldrich) and [$^2H_4$] -salycilic acid (SA) (100 ng, Sigma-Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0 using a 30% (v / v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of ethyl ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a clean vial, combining both partitions and drying them under vacuum using an evaporation centrifuge coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint-Herblain Cedex, France). The dried residue was resuspended by adding 100 μL of methanol (HPLC grade, Scharlau) in the test tube by ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure water

(MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore diameter of 0.2 μm before analysis. Phytohormone analysis was performed using an HPLC device (Alliance 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an electrospray interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and separated by a reverse phase column (Kromasil 100, C18, 5 μm, 100 × 2.0 mm, Scharlau) using a linear gradient of methanol and ultrapure water, supplemented with acetic acid to a final concentration of 0.01% (v /v) and a flow of 0.3 mL / min. Discrimination and detection of each analyte was carried out following the fragmentation pattern and the characteristic retention time. The ionization and collision conditions for each compound were optimized by direct infusion of pure standards (approximately 5 mg/L). The quantification of the analytes of interest was performed using the response factor (analytical area/area) by interpolation in a calibration curve injected alternatively in the samples. Chromatogram processing, integration and quantification were performed using MassLynx 4.0 software. The profiles of the relative content of phytohormones between strains were determined with the Heatmapper web tool (Babicki et al. 2016).

Measurement of chlorophyll fluorescence imaging from isolated algae

Fast PCR was performed directly from the unialgal cultures as described in Molins et al. (2018b) to ensure the purity and identity of the selected aliquot used for chlorophyll (Chl) fluorescence images (CFI) measurements.

A 50 ml aliquot of these actively growing algae, resuspended in liquid BBM medium, was inoculated on cellulose–acetate disks placed on agarized BBM medium and then cultured for 21 days under two conditions, 17°C and 20°C with a 50 μmol $m^{-2}s^{-1}$ PPFD under a 12 h/12 h light/dark cycle.

CFI was performed using an imaging-PAM fluorometer (Walz, Effeltrich, Germany), in order to investigate the behavior of Chl fluorescence parameters in the three algae at different light intensities and grown under different temperature conditions (17°C and 20°C). Algae samples were layered on filter paper that was kept moist with distilled water in order to maintain the cells in a fully hydrated state. The algae membranes were darkened for 30 min prior to measurement. Chl *a* fluorescence determinations were obtained from n= 4 samples for each algal strain and temperature. The minimum (dark) fluorescence (F_o) was obtained by applying measuring light pulses at a low frequency (1 Hz). The maximum fluorescence (F_m) was determined by applying a saturating blue pulse (10 Hz). The maximum quantum yield of PSII photochemistry (Rohácek 2002), often called the F_v/F_m ratio, was determined as $F_m – F_o/F_m$. Then, a light curve with actinic illumination from 0

to 964 μmol photons m^{-2} s^{-1} was switched on, and saturating pulses were applied for 20 s in order to determine the maximum fluorescence yield (F'_m) intensity, and the steady-state fluorescence value that is immediately prior to the saturating pulse is F_s. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation NPQ = $(F_m - F'_m)/F_m$ (Bilger and Björkman 1991). The actual quantum efficiency of PSII photochemistry, Φ_{PSII} , was calculated according to Genty et al. (1989) by the formula: $(F'_m - F_s)/F'_m$), and the coefficient of photochemical quenching (Van Kooten and Snel 1990) was determined as qP = $(F'_m - F_s)/(F'_m - F'_o)$. Excitation pressure on PSII (QA), which reflects the proportion of the primary quinone electron acceptor of PSII that is in the reduced state, was calculated as 1 − qP (Demmig-Adams et al. 1996). The relative electron transport rate (ETR) was calculated as $\Phi_{PSII} \times PAR \times 0.84 \times 0.5$ (Schreiber et al. 1986). To determine F'_o correctly, it would be necessary to switch off the actinic light and quickly reoxidise the PSII acceptor side with the help of far-red light, but this is not feasible with imaging-PAM as far-red light would penetrate the CCD-detector and cause serious disturbances to fluorescence images (see http://www.walz.com). The value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m + F_o/F'_m)$. For each interval, saturation pulse images and values of various Chl fluorescence parameters were captured (Calatayud et al. 2006).

Two AOI were selected one in the central part and other in the outer algae zones in order to evaluate spatial heterogeneity CFI parameters between both AOI. After comparing both AOI for each algae strain and temperature, not differences were observed for any Chl fluorescence parameters (data not shown). Then, the values of Chl fluorescence parameters displayed in the figures (4a and 5) are means of both AOI.

Statistical analyses

Data was analysed by one-way Analysis of Variance (ANOVA) followed by post-hoc comparisons by Tukey's HSD test. A probability value < 0.05 was considered statistically significant. Calculations were performed using R Software.

Results

Primary *Trebouxia* diversity detected by Sanger sequencing (nrITS)

The concatenated aligned algal nrITS + LSU rDNA fragment was 1329 bp in length. BI and ML phylogenetic hypotheses were topologically congruent. In the 46 samples, we detected three

Trebouxia species (Fig. 1). According to the clade code introduced for *Trebouxia* by Leavitt et al. (2015), and subsequently applied by Moya et al. (2017) and Muggia et al. (2020), these *Trebouxia* species belong to clade 'A' *arboricola/gigantea* type, precisely: *Trebouxia asymmetrica* in thalli from Lanzarote, *Trebouxia cretacea* in Fuerteventura, and *Trebouxia* sp. `*arnoldoi´* in Tenerife.

Ultrastructural characterization of *Trebouxia*

Transmission electron microscopy (TEM) analyses of photobionts based on the ultrastructure of pyrenoids (Py) and plastids (Chl) distinguished at least three different *Trebouxia* morphotypes. Morphological characteristics of each morphotype, in detail, can be seen in Fig. 2 and Fig. 1S.

One morphotype was found in *Buellia zoharyi* cells from Lanzarote (Fig. 2a; b). They showed a single central Py related to the gigantea type described by Friedl (1989) with pyrenoglobuli (Pg) uniformly distributed within the Py matrix. Lobulated-stellate chloroplast with abundant lax thylakoid membranes forming stacks of 4-5 membranes (grana).

The second morphotype was found in *B. zoharyi* cells from Fuerteventura (Fig. 2c, d). These cells presented a single central Py related to the impressa/gigantea type described by Friedl (1989), with a highly lobulated chloroplast with abundant dense thylakoid membranes forming stacks of 2-3 membranes.

The third morphotype was detected in *B. zoharyi* cells from Tenerife (Fig. 2e, f). They presented a single central Py related to the impressa/gigantea type described by Friedl (1989). The chloroplast morphology was similar to the second morphotype (lobulated with dense abundant thylakoid membranes forming stacks of 2-3 membranes). Large peripheral vesicles surrounding the chloroplast were present.

***Trebouxia* multiplicity revealed by specific PCR primers**

Herbarium samples were excluded from this analysis. Specific forward and reverse primers were designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia cretacea* and *Trebouxia* sp. `*arnoldoi´*

All the samples from Lanzarote (n=23) showed *T. asymmetrica* as the primary photobiont, and specific PCR detected *T. cretacea* in 22 thalli. *Trebouxia* sp. `*arnoldoi´* was not detected (Fig. 3; Table 3S). Samples from Fuerteventura (n=7) showed *T. cretacea* as the primary photobiont. The

presence of secondary algae was detected only in three thalli: two of them revealed *T. asymmetrica* as the secondary photobiont and one *Trebouxia* sp. *`arnoldoi´* (Fig. 3; Table 3S). Samples from Tenerife (n=13) had *Trebouxia* sp. *`arnoldoi´* as the primary photobiont. All the samples only showed *T. asymmetrica* as the secondary photobiont. *T. cretacea* was not detected (Fig. 3; Table 3S).

Identification and morphological characterization of isolated *Trebouxia* spp. from *Buellia zoharyi*

To corroborate the purity and identity of the unialgal selected aliquot, fast PCR was performed directly from the cultures as described in Molins et al. (2018b). Sequences obtained were included in the phylogeny indicated as isolated photobiont (Fig. 1). We detected three *Trebouxia* spp.*: T. asymmetrica, T. cretacea* and *Trebouxia* sp. *`arnoldoi´*.

All three *Trebouxia* spp. presented mature vegetative cells, mostly unicellular and spherical as seen using LM and EFM (Fig. 2S). Tetrads and octads were only observed in *T. cretacea*. The spherical vegetative cells were 14–16 (19) μm in diameter. All cells showed the characteristic *Trebouxia* central chloroplast dissected into lobes (Fig. 2S).

Phytohormone profiles of *Buellia zoharyi* photobionts

Phytohormone endogenous content was also determined to characterize *Trebouxia* strains cultured in BMM at 20º C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA, JA conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me-JA) (Fig. 4). The *Trebouxia* strains isolated from *B. zoharyi* showed differences in their phytohormone profiles:

1) *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 4a, b), *Trebouxia* sp. *`arnoldoi´*showed a different profile, with about double the ABA endogenous content compared to the other strains (Fig. 4b), and higher levels of IAA (about five times) than *T. asymmetrica* and *T. cretacea* (Fig. 4a).

2) SA and JA-Ile levels also showed a different profile between microalgae strains, with similar levels for *T. cretacea* and *Trebouxia* sp. *`arnoldoi´*, while *T. asymmetrica* showed the highest level of SA (6 times greater; Fig. 4c) and higher levels of JA-Ile than *T. cretacea* (about 400% more; Fig.4d).

3) Me-JA content of the *Trebouxia* strains showed another different profile, with similar levels for *T. asymmetrica* and *Trebouxia* sp. `*arnoldoi´*, which were about 2.6 times higher than the levels observed for *T. cretacea* (Fig. 4d).

Chlorophyll *a* fluorescence response to different temperatures (20º C and 17º C) of *Buellia zoharyi* photobionts

According to the F_v/F_m measurements (Fig. 5a), *Trebouxia* sp. `*arnoldoi´* was significantly affected by temperature, with the lowest F_v/F_m values at 17º C. Similarly, in *T. cretacea*, F_v/F_m showed a significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17º C However, *Trebouxia asymmetrica* showed similar values at 20º C and 17º C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them. The highest F_v/F_m values were obtained for *T. asymmetrica* and *T. cretacea* at 20º C.

The observation of color changes, ranging from black (0.000) to pink (1.000), revealed that the images for F_v/F_m showed a uniform color for algae discs, and that different intensities of blue colors associated with higher values of Fv/Fm (Fig. 4b) for *T. asymmetrica* and *T. cretacea* at 20º C, and with the lowest F_v/F_m values (green) for *Trebouxia* sp. `arnoldoi´ at 17º C (Fig. 5b).

All NPQ values increased with respect to light intensity, but with a different shape and magnitude depending on the *Trebouxia* species and growth temperature (Fig. 6a). In the case of *Trebouxia* sp. `*arnoldoi´* and *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes showing the same kinetic shape, but *Trebouxia* sp. `*arnoldoi´* showed the highest NPQ values. *T. cretacea* at 20º C and *T. asymmetrica* displayed similar NPQ values and at the end of light-curve kinetics *T. cretacea* showed the highest NPQ values.

Regarding ETR (Fig. 6b), their values were slightly higher for *Trebouxia* strains growing at 20 º C compared with 17º C. Among species, *T. cretacea* displayed the highest ETR values, mainly at the end of light curve kinetic at 20º C and the minimum values were observed in *Trebouxia* sp. `*arnoldoi´* at 17º C.

Photosystem II excitation pressure, expressed as 1-qP (photochemical quenching; Fig. 6c), increased gradually with light intensity in the three algal strains. In general, *T. asymmetrica* and *T. cretacea* showed similar values, however *Trebouxia* sp. `*arnoldoi´*showed slightly values except at high irradiance where *Trebouxia* sp. `*arnoldoi´*at 17º C showed the highest values.

Discussion

This study applied a multidisciplinary approach to describe microalgae diversity from *B. zoharyi* growing in the Canary Islands. Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the region, and suggest that bioclimatic factors could influence the myco/photobiont association patterns.

According to Beck et al. (2002), 'selectivity' in lichens refers to the taxonomic range of partners that are selected by one of the bionts, while 'specificity' should be used for the symbiotic association, and depends on the range and taxonomic relatedness of acceptable partners. Lichens with high selectivity may associate with a limited number of photobionts. Numerous lichen-forming fungi have been shown to associate with identical species of *Trebouxia*, while others exhibited higher photobiont flexibility (Kroken and Taylor 2000; Ohmura et al. 2006, 2018; Doering and Piercey-Normore 2009; Leavitt et al. 2013, 2015; Lindgren et al. 2014). Our results indicate that *B. zoharyi* are flexible in their photobiont choice, as they associate with three *Trebouxia* species in 300 km (distance from Igueste de San Andrés; Tenerife to Los Valles; Lanzarote).

Photobiont diversity can be shaped by the reproductive and dispersal strategies of the mycobiont (Cao et al. 2015, Steinová et al. 2019), geography (Muggia et al. 2014, Werth and Sork 2014, Leavitt et al. 2015), growth substrate (Bačkor et al. 2010, Leavitt et al. 2013, Muggia et al. 2014) and macroclimate (Lu et al. 2018, Singh et al. 2018). Lichens that reproduce sexually via independent dispersal of fungal spores, undergo a process of re-lichenisation. This means that the germinating spore of the mycobiont can easily exchange its autotrophic partner, in contrast to asexually reproducing lichens distributing both partners together, which allows the continuation of the symbiosis without the need to re-associate with another biont (Beck et al. 1998, 2002; Romeike et al. 2002; Sanders and Lücking 2002). Lichens that depend on the cyclical establishment of fungal-photobiont associations to colonize varied wide-ranging habitats might require a relatively higher flexibility in the specificity and ecological selection of their photobionts. This flexibility would facilitate successful re-lichenizations by allowing for alternative partnerships in each habitat (Romeike et al. 2002

The dispersal of *B. zoharyi* over medium to long distances can be accomplished by either meiotic ascospores produced in fertile thalli, or thallus fragments detached from fertile and sterile ones (Barreno 1994; Casares and Llimona 1983). Both strategies are suitable for long- distance dispersal, even across the Mediterranean Sea, based on evidence of other lichens showing widely disjunct populations (Alors et al. 2017; Fernández- Mendoza and Printzen 2013; Garrido-

Benavent et al. 2018). Moreover, increased connectivity among the Canary Islands, Africa and the Iberian Peninsula possibly occurred during Pleistocene glaciations, when the distance between the Eastern most island (Fuerteventura) and the Moroccan coast was reduced to 60 km (Fernández-Palacios and Whittaker 2008). Chiva et al. (2016) showed *T. cretacea* as the predominant photobiont in 117 thalli of *B. zoharyi* from the Iberian Peninsula and Morocco. The colonization of the Canary Islands by *B. zoharyi* must have originated from the Moroccan coast to Fuerteventura (20.7 Ma), currently the eastern island, only 100 km from the African coast, evidenced as the maintenance of the symbiont pattern (*T. cretacea*). Carracedo (1994), dated the origin of Lanzarote in 15.5 Ma and Tenerife in 11.6 Ma. *B. zoharyi* must have colonized the Canary Islands subsequently and adopted ecologically adapted photobionts (*T. asymmetrica* and *Trebouxia* sp. `*arnoldoi´*) in those Islands.

The diversity of photobionts has only recently been explored by environmental DNA metabarcoding approaches, and has focused on species within the Mediterranean basin to date (Moya et al. 2017; Dal Grande et al. 2018; Smith et al. 2019). In contrast to high-throughput sequencing approaches, traditional and largely applied DNA barcoding using Sanger sequencing was able to detect only the principal photobiont in the thalli (Paul et al. 2018; Moya et al. 2020). In this study, the authors determined if the second most abundant microalga exceeded 30% of the total HTS reads in a sample, Sanger sequencing generally failed and generated ambiguous Sanger sequences showing double peaks. Moreover, in the present study no samples with double peaks were found in the electropherogram, the co-occurrence of multiple *Trebouxia* inside a thallus was performed by using specific primers. This approach may limit the detection of further associated algae due to specificity and thus, it should be used as a complement to traditional Sanger sequencing when it is not possible to perform HTS approaches. However, all analyses performed using the multi-copy nrITS showed methodological limitations that potentially bias the results presented, due to the variation in the copy numbers across microalgae species. Therefore, the relative abundance of algal groups inferred in this study with specific primers does not accurately describe the true relative abundance of lichen-associated algae, given the potential for a very wide range of nrDNA copy numbers of these algal groups. Even with these limitations, specific PCR primers revealed the presence of other secondary photobionts which would be available in the substrate. The presence of multiple algal species, and the different dominance of one of them in each species, implies the selection for a particular algal species by the mycobiont (Peksa and Škaloud 2011; Dupont et al. 2016). To corroborate this hypothesis, a complementary HTS approach, both from the lichen and from the substrate, should be included.

A reliable definition of photobiont species requires the description of morpho-anatomical traits both axenically cultured and in symbiosis. However, such traits are absent for the majority of lineages described in molecular phylogenetic analyses (Muggia et al. 2020). In this study, photobionts from Lanzarote, Fuerteventura and Tenerife were characterized in *Buellia zoharyi* thalli using transmission electron microscopy (TEM). The three morphotypes structurally characterized corresponded to each *Trebouxia* species molecularly described for each Island. These three *Trebouxia* species belong to clade 'A' *arboricola/gigantea* (Fig. 1) and showed, by TEM, a similar pyrenoid type (Fig. 2 and Fig. 1S), corresponding to the *impressa/gigantea* type described by Friedl (1989). Although Lanzarote´s morphotype showed a high similarity to the *gigantea* type, this trait does not allow us to differentiate each morphotype. In contrast to the pyrenoid, the chloroplast thylakoid arrangements clearly differentiated the three morphotypes. This coincides with further studies (Molins et al. 2018a) and evidences that the thylakoid arrangement is a key complement to the pyrenoid structure to characterize *Trebouxia* species. Current knowledge concerning *Trebouxia* phylogenetic relationships highlights the pressing needs to revise the original classification of the group proposed by Friedl (1989) as that classification does not match with Muggia et al.'s (2020) clade delimitation. The combination of molecular analyses together with ultrastructural techniques should be initiated to clarify taxonomic concepts to delimit new taxa of microalgae, and particularly in the case of *Trebouxia* diversity (Muggia et al. 2016, 2020; Moya et al. 2017; Molins et al. 2018b). Here we detected the new *Trebouxia* sp. *'arnoldoi'* which did not match any previously described *Trebouxia* spp. This provisional name has been proposed until it can be formally described: `arnoldoi´refers to the Canarian botanist Arnoldo Santos.

Isolation procedures should be included in photobiont diversity studies to perform morphological and physiological analyses. In this study, the *Trebouxia* strains isolated from *B. zoharyi* were visualized from unialgal cultures using light microscopy (LM) and epifluorescent microscopy (EFM) and its phytohormone composition has been included to characterize them. This technique has been recently performed by Pichler et al. (2020) with the lichen forming algae *Trebouxia* sp., *Trebouxia decolorans* and *Asterochloris glomerata*. Under controlled experimental conditions, *T. cretacea*, *T. asymmetrica* and *Trebouxia* sp. *`arnoldoi´* showed characteristic profiles of endogenous content in phytohormones (Fig. 4). IAA levels found in *Trebouxia* sp.*'arnoldoi'* (Fig. 4a) are similar to those observed in *Trebouxia* sp., *T. decolorans* and *A. glomerata* grown in solid media (Pichler et al. 2020). ABA production has been also detected in in these three microalgae (Pichler et al. 2020) as well as in *Trebouxia* sp. TR9 (Hinojosa-Vidal et al. 2018) isolated from *Ramalina farinacea* (Pichler et al. 2020). The ABA levels observed in the *Trebouxia* strains tested in this study (Fig. 4b) are in the same range of magnitude as the levels in *Trebouxia* sp. TR9, *Trebouxia* sp. and *T.*

decolorans (Hinojosa-Vidal et al. 2018; Pichler et al. 2020). In contrast, the endogenous bioactive form of JA, JA-Ile (Fonseca et al. 2009), was found in all three *Trebouxia* strains from *B. zoharyi* (Fig. 4d), while no detectable endogenous levels of JA have been found by Pichler et al. 2020.)In addition, SA and Me-JA were also detected in the *Trebouxia* strains isolated in this study but they were not previously described in lichen-forming algae, although Me-JA has been detected in *Chorella* (Ueda et al. 1991).

In conclusion, the different phytohormone profiles obtained in the three *Trebouxia* strains isolated in this study (Fig. 4f), and in the other lichen forming algae mentioned above, suggest that each microalgae strain could present different internal signalling needs. On the other hand, it cannot be ruled out that a part of the production of these phytohormones could also be secreted to the external environment and play a role in external signalling mechanisms with the other members of the thallus symbiotic system. Related to this, the extracellular release of IAA, ABA and JA in the lichen-forming algae *Trebouxia* sp., *T. decolorans* and *Asterochloris glomerata*, has recently been described (Pichler et al. 2020). The determination of the role of these phytohormones in the internal signalling mechanisms of the photobionts, as well as in the coordination mechanisms with the rest of the elements of the lichen symbiosis, is a question that needs future studies.

The worldwide distribution of lichen species was also hypothesized to be strongly correlated with the ecological specialization and the physiological performances of the photobionts (Casano et al. 2011; Peksa and Škaloud 2011). In lichens, different degrees of mycobiont-photobiont specificity is known (Leavitt et al. 2015; Beck et al. 2019; Steinová et al. 2019) and in some cases these relationships have been correlated to the suitability and the physiological performance of the photobionts in diverse, or even changeable, environmental settings (Vančurová et al. 2015; Steinová et al. 2019).

The lichen *Ramalina farinacea* (L.) Ach. has proved to be a suitable reference and model species for studying microalgal diversity, as it recurrently shows the co-occurrence of at least two photobionts (*Trebouxia* sp. TR9 and *T. jamesii*) inside the thalli using microscopy techniques, culture isolations and molecular characterization with different genetic markers (del Campo et al. 2010b, 2013). Moreover, the predominant photobiont differs between different populations; *T. jamesii* being the most abundant in the Iberian Peninsula and *Trebouxia*. sp. TR9 in the Canary Islands. Several studies have further demonstrated that these two photobionts respond differently to abiotic stresses (Casano et al. 2011). Casano et al. (2011) also analysed the effects of temperature and light on the growth and the photosynthetic traits of isolated photobionts from *R. farinacea* (*Trebouxia* sp. TR9 and *T. jamesii*). They found that both species grew better at the lowest temperature and performed the experiments only in cultures grown at 17º C. On the contrary, we did

not find such differences in the growth temperature, and thus, we performed the experiments at both 17º C (mean annual temperature in Fuerteventura and Lanzarote) and 20º C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures **(**20º C and 17º C**)**.

The maximum quantum yield of primary photochemistry in dark-adapted leaves (F_v/F_m) in healthy plants reach F_v/F_m values around 0.840, but for lichens this parameter ranges between 0.550 and 0.700 (Casano et al. 2011; Gasulla et al. 2019). In this study F_v/F_m values ranged between 0.51-0.66. The decline in F_v/F_m might be a result of an increase in non-photochemical processes in the light harvesting antennae of PSII associated with a photochemical quenching down-regulation, photodamage of PSII reaction centres, or both (Osmond et al. 1993). In this study, we observed that *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In contrast, *Trebouxia* sp. *`arnoldoi´*and *T. cretacea* were more sensitive to changes in growing temperature, showing a significant decline in F_v/F_m when grown at 17º C with respect to 20º C. In addition, these species showed the lowest ETR, mainly from 400µmol photons m^{-2} s^{-1} to the end of light curve. The electron transport rate was saturated at approximately the same irradiance (700 µmol photons m^{-2} s^{-1}) in all *Trebouxia* species (except *T. cretacea*) under both temperatures; similar results were obtained in *Trebouxia* sp. TR9 and *T. jamesii* (Casano et al. 2011). A decrease in ETR and an increase in 1-qP indicate a reduction in the quantum yield of PSII and a reduction state of the first electron acceptor of PSII, QA, respectively. In this circumstance, non-photochemical processes (NPQ) must be increased to guarantee excitation energy dissipation (Havaux et al. 1991) such as occurred in *Trebouxia* sp. *`arnoldoi´* where higher NPQ and 1-qP, and the lowest ETR, was observed. NPQ plays an important role in plants against excess radiation. In these algae strains from *Buellia zoharyi,* NPQ increases in all of them reducing the excitation pressure on reaction centres, thereby decreasing the possibility of photodamage (Papageorgiou and Govindjee 2014). These different behaviours between algae strains grown under two temperatures have been observed by Casano et al. (2011) in *Trebouxia* sp. TR9 and *T. jamesii* where *Trebouxia* sp. TR9 performed better under high temperatures, indicating a different capacity to adaptation.

Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the region, and suggest that bioclimatic factors could influence the myco/photobiont association patterns due to the different photosynthesis response to different temperatures **(**20º C and 17º C**).**

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Fig. 1 *Trebouxia* phylogenetic analysis. Rooted ITS1-5.8S-ITS2 + LSU rDNA gene tree representing 41 *Trebouxia* sequences, including 28 well- accepted *Trebouxia* species from SAG and UTEX, *Trebouxia* sp. TR9 and *Trebouxia crespoana* from ASUV retrieved from the GenBank. Newly generated sequences are marked as ex. *Buellia zoharyi*_locality_haplotype code_frequency. Three *Trebouxia* species detected were indicated. Values at nodes indicate statistical support estimated by two methods: bootstrap support (BS, RAxML analysis) and posterior probabilities (PP, MrBayes analysis). Scale bar shows the estimated number of substitutions per site.

Fig. 2 a, b Cross-section of *B. zoharyi* thalli from Lanzarote by TEM. **a** Photobionts of *B. zoharyi* inside thallus, **b** Detail of pyrenoid. **c, d** Cross-section of *B. zoharyi* thallus from Fuerteventura by TEM. **c** Photobionts of *B. zoharyi* inside thallus, **d** Detail of pyrenoid. **e, f** Cross-section of *B. zoharyi* thalli from Tenerife by TEM. **e** Photobionts of *B. zoharyi* inside thallus, **f** Detail of pyrenoid. Abbreviations: Py, Pyrenoid; Pg, Pyrenoglobuli; Chl, Chloroplast; PV, Peripheral vesicles. Bars 500 nm, 800 nm, 1 µm and 2 µm.

Fig. 3 Algal multiplicity detected by PCR depicted in the localities included in this study: Tenerife, Lanzarote and Fuerteventura. Inner, outer circle and numbers represent the primary and secondary

photobiont detected, respectively. The colour coding for each *Trebouxia* is shown on the bottom left-hand side of the figure.

Fig. 4 Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20º C. Three cultures were sampled for each condition and phytohormone levels were quantified: **a** indole-3-acetic acid (IAA); **b** abscisic acid (ABA); **c** salycilic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and **e** methyl jasmonate (Me-JA). Graphs show the mean ± standard deviation. Significant differences between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis of the phytohormone content of each Trebouxia strain. Scale bar (Z-score) indicates the relative abundance of a particular phytohormone in each strain.

Fig. 5 a Maximum fluorescence yield (F_v / F_m) of the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. Bars represent means ± SE, n = 4. Significant differences between treatments and species are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **b** Chlorophyll fluorescence images for Fv/Fm of the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. The color scale bar shown at the bottom of the figures stands for values from 0 (black) to 1 (pink).

Fig. 6 a PPFD response curves of the non-photochemical quenching (NPQ), **b** relative electron transport rate (ETR), and **c** PSII photooxidative pressure (1-qP) in the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. Bars represent means ± SE, n = 4.

Fig

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Fig

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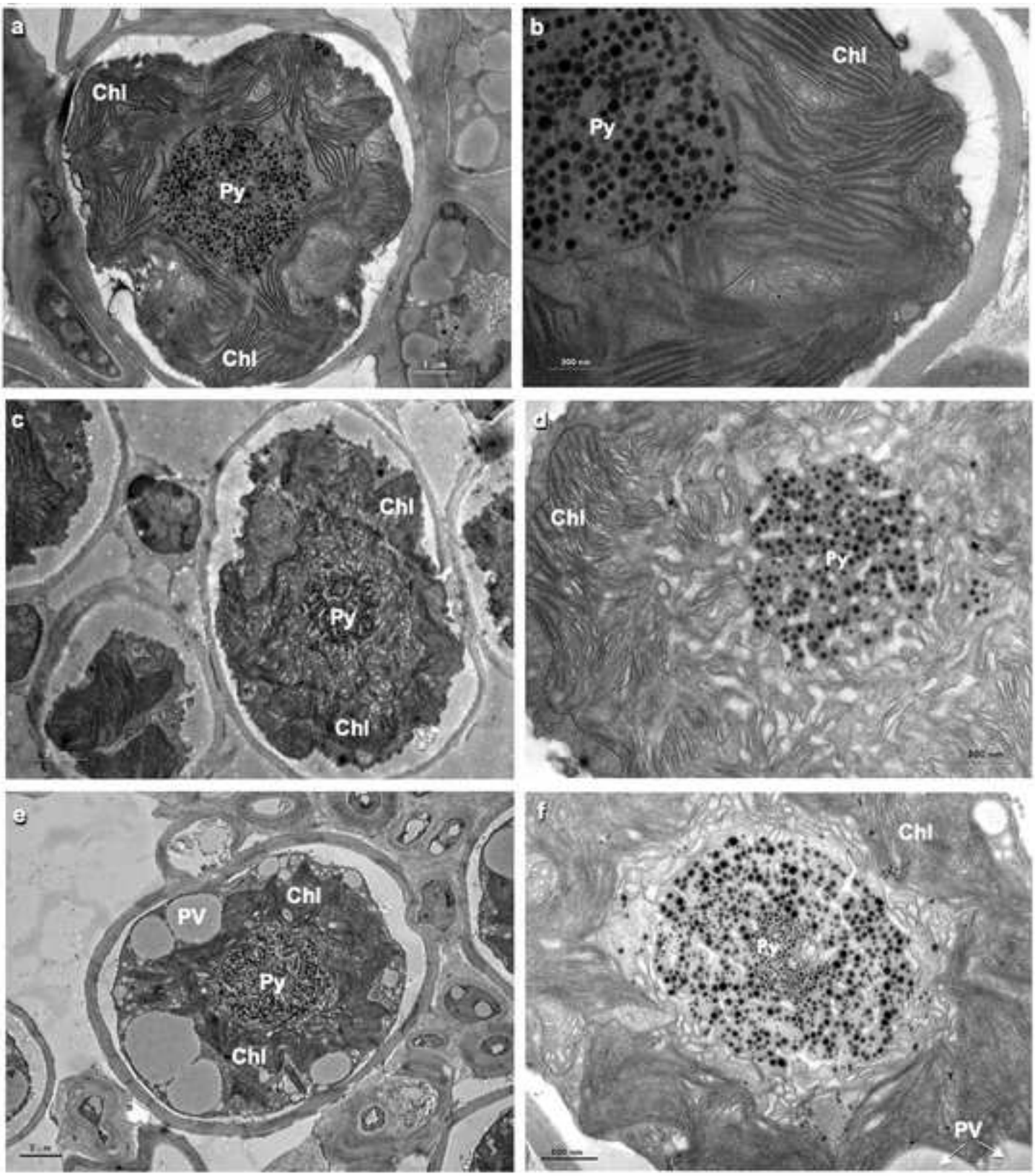

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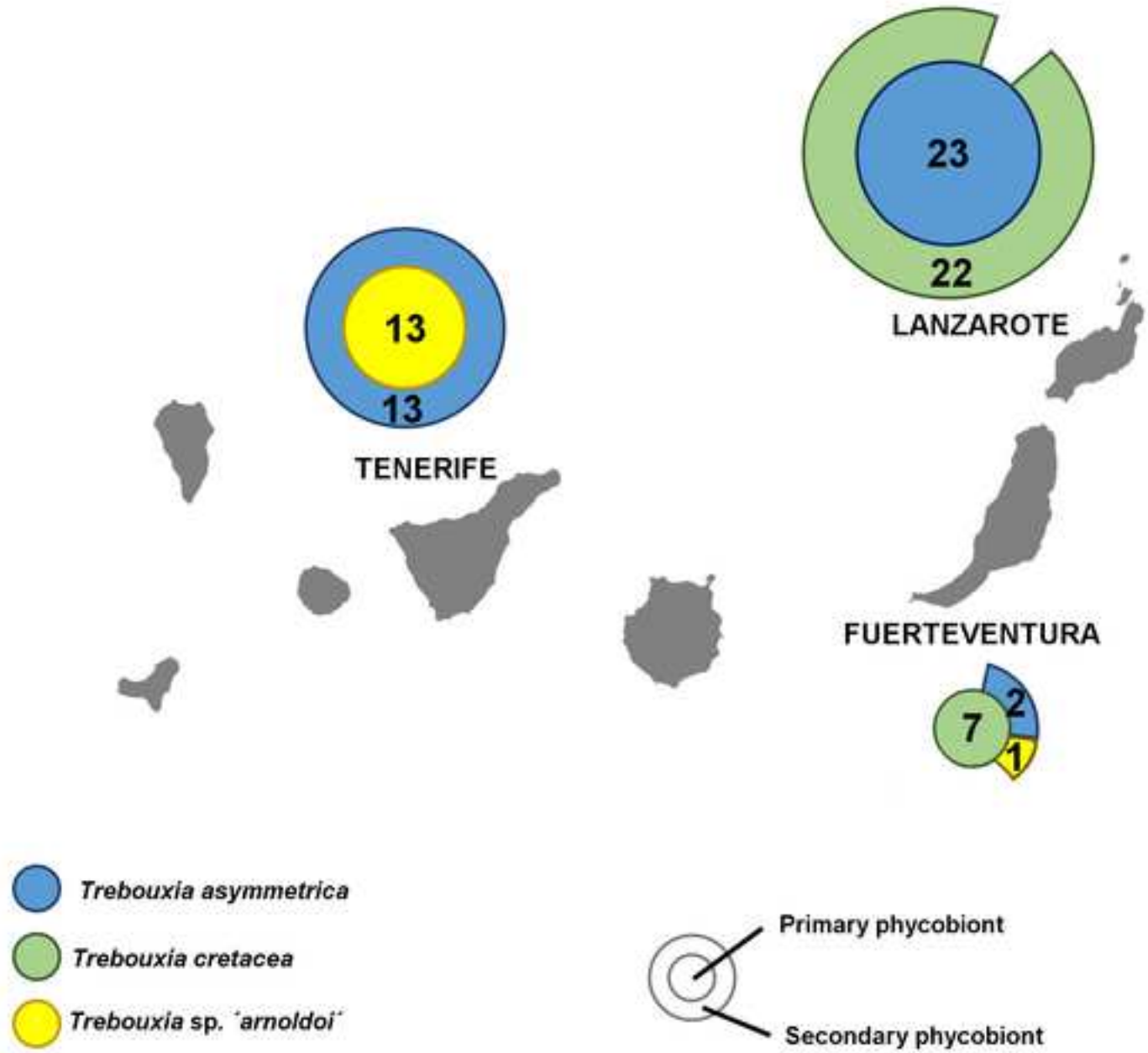

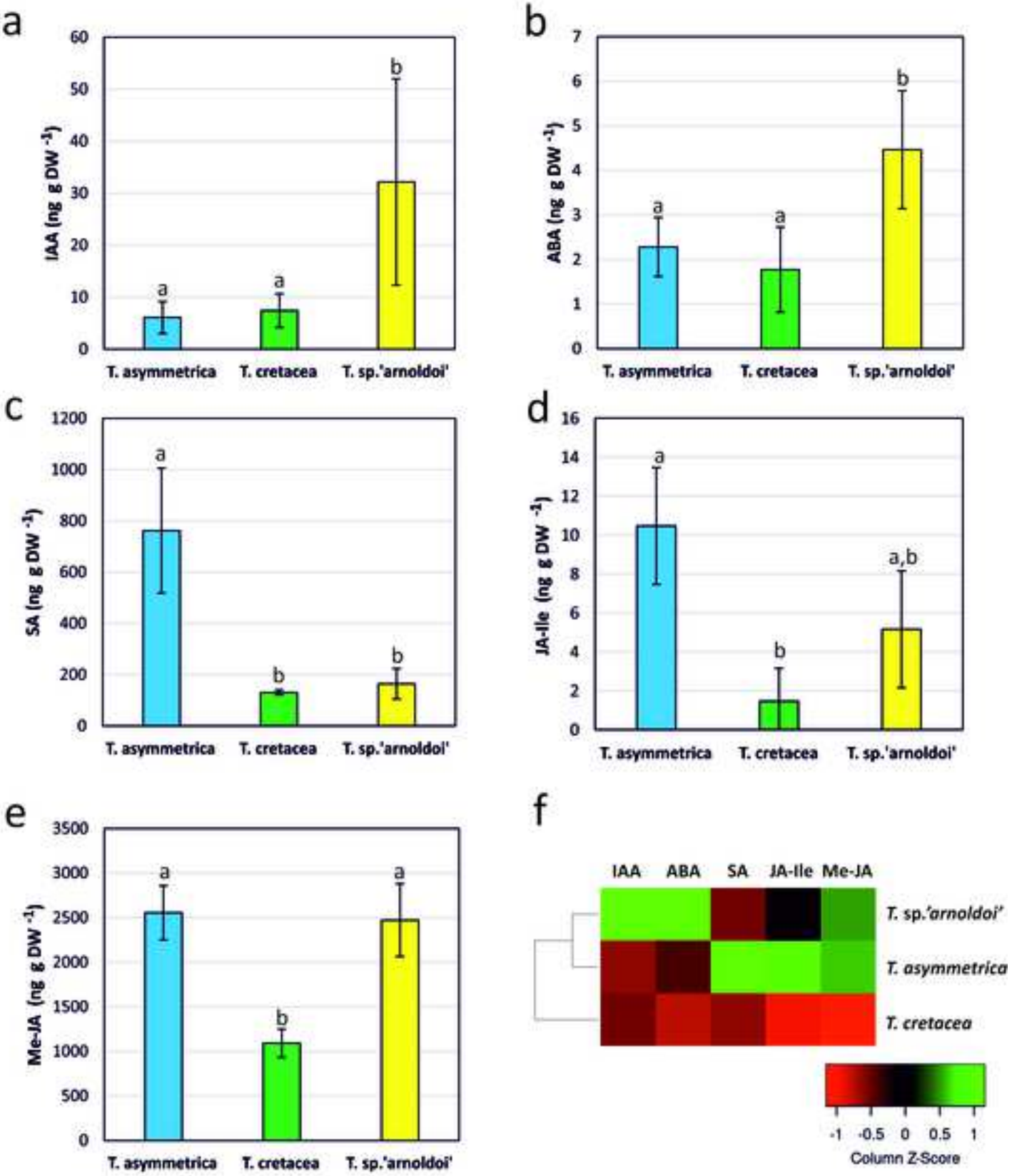

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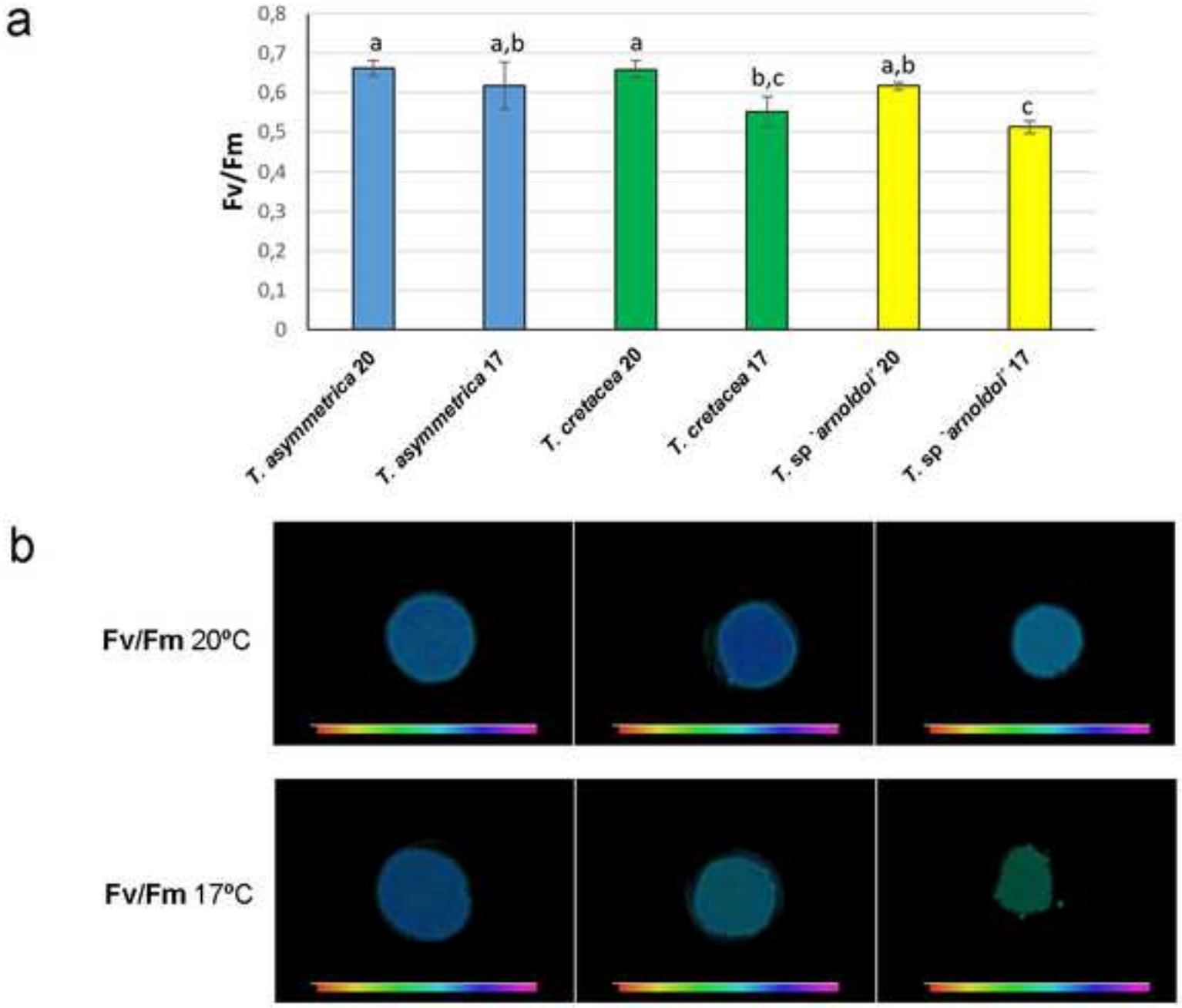

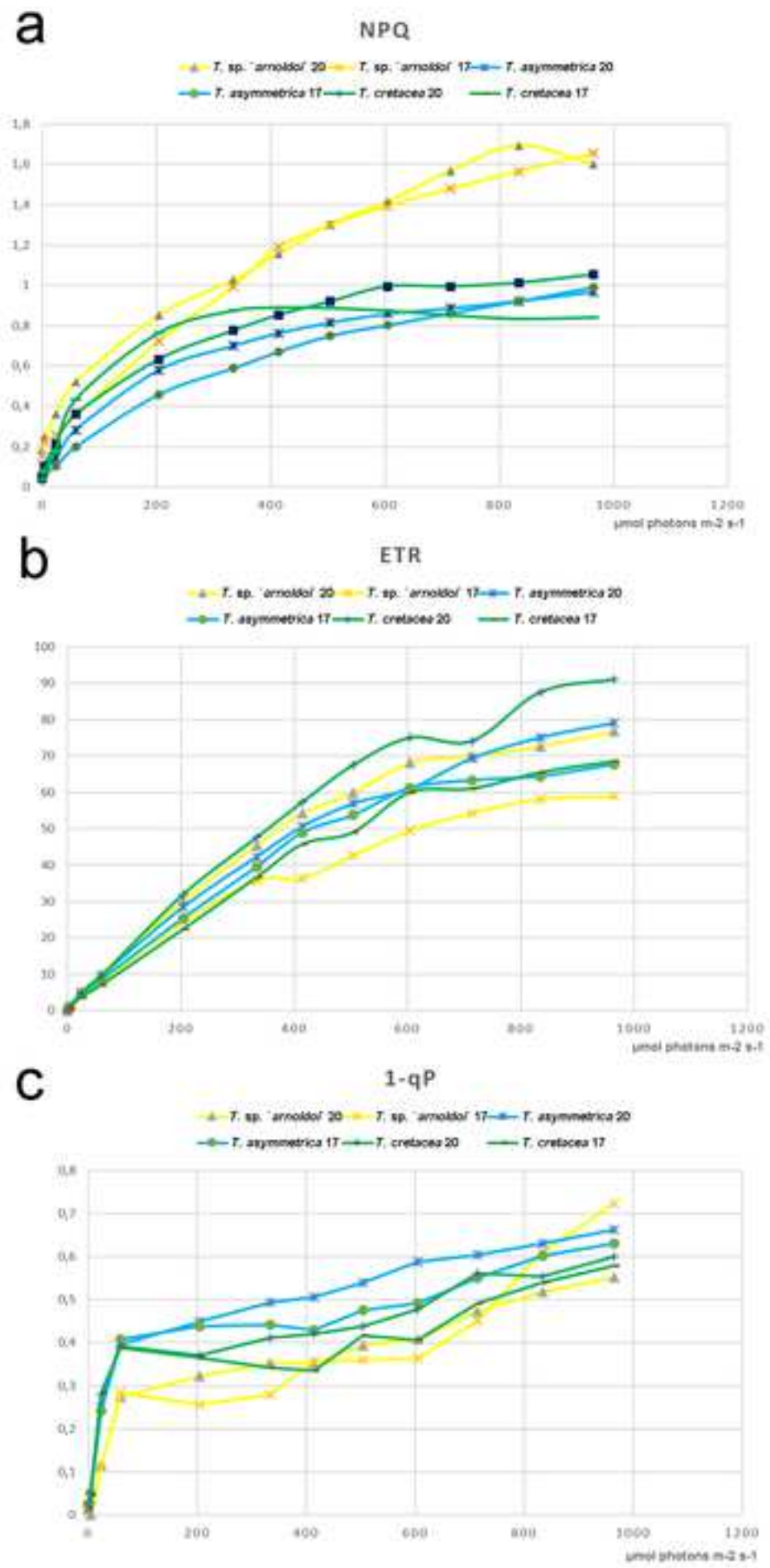

Table sup

		Primary phycobiont
Locality	Sample code	LSU rDNA
Lanzarote, Los Valles	LA 1	T. asymmetrica
Lanzarote, Los Valles	LA 2	T. asymmetrica
Lanzarote, Los Valles	LA 3	T. asymmetrica
Lanzarote, Los Valles	LA 4	T. asymmetrica
Lanzarote, Los Valles	LA 5	T. asymmetrica
Lanzarote, Los Valles	LA 6	T. asymmetrica
Lanzarote, Los Valles	LA 7	T. asymmetrica
Lanzarote, Los Valles	LA 8	T. asymmetrica
Lanzarote, Los Valles	LA 9	T. asymmetrica
Lanzarote, Los Valles	LA 10	T. asymmetrica
Lanzarote, Los Valles	LA 11	T. asymmetrica
Lanzarote, Los Valles	LA 12	T. asymmetrica
Lanzarote, Los Valles	LA 13	T. asymmetrica
Lanzarote, Los Valles	LA 14	T. asymmetrica
Lanzarote, Los Valles	LA 15	T. asymmetrica
Lanzarote, Los Valles	LA 16	T. asymmetrica
Lanzarote, Los Valles	LA 17	T. asymmetrica
Lanzarote, Los Valles	LA 18	T. asymmetrica
Lanzarote, Los Valles	LA 19	T. asymmetrica
Lanzarote, Los Valles	LA 20	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 21	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 22	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 23	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 24	T. asymmetrica
Lanzarote, Haria, Peñas del Chache	LA 25	T. asymmetrica
Tenerife, Igueste de San Andrés	TE 1	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 2	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 3	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 4	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 5	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 6	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 7	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 8	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 9	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 10	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 11	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 12	T. sp. `arnoldoi´
Tenerife, Igueste de San Andrés	TE 13	T. sp. `arnoldoi´
Fuerteventura, Betancuria	FU 1	T. cretacea
Fuerteventura, Betancuria	FU 2	T. cretacea
Fuerteventura, Betancuria	FU 3	T. cretacea
Fuerteventura, Betancuria	FU 4	T. cretacea
Fuerteventura, Betancuria	FU 5	T. cretacea
Fuerteventura, Betancuria	FU 6	T. cretacea
Fuerteventura, Betancuria	FU 7	T. cretacea

Fuerteventura, Betancuria	FU 8	T. cretacea

		Specific PCR		
nrITS	Coexistence	T. cretacea	T. asymmetrica	T. sp. arnoldoi
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	NO	absent	present	absent
T. asymmetrica	YES	present	present	absent
T. asymmetrica	NOT ANALYZED (HERBARIUM)			
T. asymmetrica	NOT ANALYZED (HERBARIUM)			
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. sp. `arnoldoi´	YES	absent	present	present
T. cretacea	NO	present	absent	absent
T. cretacea	YES	present	present	absent
T. cretacea	YES	present	present	absent
T. cretacea	NO	present	absent	absent
T. cretacea	NO	present	absent	absent
T. cretacea	YES	present	absent	present
T. cretacea	NO	present	absent	absent

T. cretacea	NOT ANALYZED (HERBARIUM)			

Cover letter

Ref: Ms. No. SYMB-D-20-00159

Title: Temperature as a bioclimatic factor affecting association patterns in *Buellia zoharyi* from the Canary Islands

Note for the Editor: Dear David there was a mistake related to the author´s order in the submission menu, the correct order is the one that appear in the doc. Sorry for this inconvenience

Authors: Arantzazu Molins[1], Salvador Chiva[1], Ángeles Calatayud[2], Francisco Marco[3], Francisco García-Breijo[4,5], José Reig-Armiñana[1,4], Pedro Carrasco[3], Patricia Moya[1]

Editor comments

Please consider the comments and recommendations for revision by the Reviewers. The recommendations for changes, though in some cases substantial, are well worth making as I am sure the paper will be much improved as a consequence. If there are any recommendations you do not wish to comply with, please identify these and state your reasons in an E-mail with the attached electronic version of your revised manuscript.

Thank you very much for your recommendations. Substantial changes have been made in the manuscript. See def version and with CC attached

Reviewer Comments:

Reviewer 1

Line 93: check ever – present

Done. See line 92

Line 120: occur instead of occurs

Done. See line 116

Line 253 algal strain instead of algae strain

Done. See line 277

Reviewer 2

I would suggest that this be published without the additional physiological measurements. These need to be much more intensively and carefully studied before we can know if the observed patterns have anything to do with the ability of the strains to display differences in acclimation and adaptation to temperature.

Thank you very much for your recommendations. We have reorganized, reduced and re-written the complete manuscript. We decided not to exclude the physiological measurements but we have changed the approach of this part of the study, because as the referee mentioned additional measurement should be included to confirm the influence of the temperature in the photobiont adaptation. However, we consider that each *Buellia zoharyi* photobionts showed different Chlorophyll *a* fluorescence responses to different temperatures (20º C and 17º C) as according to the F_v/F_m measurements (Fig. 5a). As shown in this Fig. *Trebouxia* sp. *`arnoldoi´* and *T. cretacea* were significantly affected by temperature, with the lowest F_v/F_m values at 17º C. However, *Trebouxia asymmetrica* showed similar values at 20º C and 17º C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them. Therefore, those differences are described in the manuscript.

I certainly don't believe that the data presented justify the title of the m/s: "Temperature as a bioclimatic factor affecting association patterns in Buellia zoharyi". I personally would doubt if temperature has much influence, but in any event, much more work is needed.

As the referee recommended we have changed the title: Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia zoharyi* from the Canary Islands

Briefly, my feeling is that the differences in temperature between the sites (17 and 20oC) are too small to have selected different photobionts. Studies that have focused on thermal adaptation have typically used populations of lichens growing in areas with much greater differences in temperature. For example, Sahu et al. (2019) measured NPQ etc., in Phaeophyscia hispidula and Flavoparmelia caperata growing along a large altitudinal gradient, with average temperatures between sites varying by more than 20oC. In the present study with Buellia, differences in photobiont composition would seem to be unlikely to be related to such small differences in temperature when other factors e.g. N supply may vary between sites.

As mentioned before, we agree with the referee that additional measurement should be included to confirm the influence of the temperature or other bioclimatic factors in the photobiont adaptation hypothesis.

We know, Sahu et al (2019) studied variations in microclimatic attributes and their effects on photosynthetic efficiency and analyzed four elevational ranging from 1950 to 3508 m. They detected that extreme variations in air temperature (5.75–31. 65º C) and other factors were imperative in controlling species richness, distribution and photosynthetic quenching of lichen flora in the region. However, in this work, *Buellia zoharyi* populations included in this study grown in areas with similar ecological conditions except for the temperature although other factors could be influence on their performance

Moreover Casano et al (2011), as mentioned in the manuscript (see lines 527-539). To search for physiological differences between the two *R. farinacea* phycobionts, studied the effects of temperature and light on the growth and photosynthetic traits of isolated and cultured *T. jamesii* and *Trebouxia* sp. TR9 algae. They showed the fresh weight reached by both microalgae after 30 days at 17°C or 20°C and 15–100 $\mu molm^{-2}s^{-1}$ of PAR. Both species grew better at the lower temperature; however, at 20°C the negative impact of the high temperature was less on TR9 than on TR1, whose growth was almost inhibited. In fact, since the low algal biomass at 20°C significantly increased the experimental error of fluorescence measurements, rendering non- reliable data, so the experiments in Casano et al (2011) were carried out only in cultures growing at 17°C. Therefore, the influence of only 3-4 ºC of temperature in the *Trebouxia* spp. was previoulsy reported by the mentioned authors.

On the contrary, we did not find such differences in the growth temperature, and thus, we performed the experiments at both 17º C (mean annual temperature in Fuerteventura and Lanzarote) and 20º C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures **(**20º C and 17º C**)**.

In the same way, I cannot accept that experiments that quantified the responses of the photobionts to the two temperatures can explain the observed differences in photobiont composition. Thus, while it is interesting that strains cultured at 17oC compared with 20oC display slightly lower Fv/Fm values, it is hard to really interpret this observation without more data. It certainly does not correlate simply with NPQ values.

See previous answer

From the data in Figure 5, it appears that algae cultured at 17oc have slightly higher ETR rates, which is not consistent with the Fv/Fm data. In general, I found the logic in the paragraphs discussing the fluorescence data muddled and difficult to follow, and furthermore, does not refer back to the figures. But ultimately, based on the data presented, photobiont composition cannot be related to the response of the photobionts to temperature.

We have rewritten this part of the document to clarify it

I am rather skeptical that individual spot measurements of phytohormone levels will tell us much about the stress tolerance / differences in the different photobionts. The work of Pichler et al. (2020) cited by the authors illustrates one possible approach. Here, first secretion of hormones was studied, and second changes in the levels of the hormones induced by light and desiccation. In theory, a study comparable to this with the different strains / geographical isolates could be valuable, but the data presented are not helpful. In conclusion therefore, my recommendation therefore is that the physiology sections are deleted and the m/s be re-submitted without them.

Thank you very much for your recommendations. Due to the scarce information related to the phytohormone content in microalgae available in the literature, we agree with the referee that a study comparable to Pichler et al. (2020) with different strains / geographical isolates exposed to several growth conditions (control and stress), would be valuable to understand photobiont adaptation, given the well know relationship between phytohormones and adaptation to environmental conditions. However, in the present study this analysis was included as a preliminary technique in order to complement the photobiont characterization. In this sense, we have been able to detect different phytohormone profiles for the three isolated photobionts, an observation that could suggest different signaling needs for each of them. We consider this data an interesting start point for further studies that could help to determine how phytohormone signaling could contribute to explain the local adapted photobiont hypothesis. In order to clarify our point of view to a future reader, we have reordered the information regarding phytohormone data in material and methods, results and discussion in the manuscript.

Minor Comments

In general replace phycobiont with photobiont

Done. See new manuscript

Line 99 Meaning of "outcompete" not clear

We have changed adapt and outcompete for thrive. See line 98

Line 123 Meaning of "communities" not clear - "populations"?

Done. See line 119

Line 126 something is missing before "population"

Done. See line 122

Line 127 "have" rather than "showed"

Rephrased. See lines 123-125

Line 128 a "biocrust" is not an ecological condition

Rephrased. See line 125

Line 139 "document" rather than "depict"

We have modified depict for describe. See line 136

Line 144 "They were characterized by means of molecular, microscopy and spectrometry." Please turn into a grammatical sentence.

Done. See line 141

Line 291 "grau"?

Grade. See line 247

Line 306 replace "with" with "with the"

Done. See line 262

Line 402 First paragraph of the Discussion should summarize the findings of the study, not the aims!!

Thank you for the suggestion. We have rewritten these paragraph. See lines 406-409

Line 420 better give area in square km

Sentence rewritten. See lines 418-419

Line 486 "resolutive", odd word, please re-phrase

Sentence deleted.

Line 538 "heterogeneities of Chl fluorescence" - is this data really used? If not, maybe delete reference to it.

We agree with the referee. Thank you for the recommendation. We have deleted this paragraph and reference

Line 554 "which could indicate sensitivity to photoinhibition" - rather an odd statement, given that the photobionts were cultured at 50 umol m-2 s-1?

Sentence deleted

Line 937 Caption to Figure 5. "Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas." This text seems to be in the wrong place?

Paragraph included in material and methods. See lines 299-303

Journal: Symbiosis

Article type: Special Issue Festschrift Barreno

Title:

Multidisciplinary approach to describe *Trebouxia* diversity within lichenized fungi *Buellia zoharyi* from the Canary Islands

~~**Temperature as a bioclimatic factor affecting association patterns in *Buellia zoharyi* from the Canary Islands**~~

Authors: Arantzazu Molins[1], Salvador Chiva[1], Ángeles Calatayud[2], Francisco Marco[3], Francisco García-Breijo[4,5], José Reig-Armiñana[1,4], Pedro Carrasco[3], Patricia Moya[1]

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Field Code Changed

Running title: ***Trebouxia* species within *Buellia zoharyi* from the Canary Islands**

Abstract

The Canary Islands are famous for their extraordinary biodiversity; however, lichenized algae have only been studied partially. *Buellia zoharyi* is a circum-Mediterranean/Macaronesian species that usually occurs in semi-arid areas of the Mediterranean, but occasionally some interesting communities of this species grow on basaltic lava flows in Lanzarote, Fuerteventura and Tenerife. Those three locations showed similar ecological conditions, but different mean annual temperatures. Here we applied a multidisciplinary approach to describe~~pict~~ microalgae diversity from *B. zoharyi* covering the entire described range of distribution in the Canary Islands. ~~Phycobiont~~Photobionts were characterized in symbiosis ~~by means of~~using molecular and microscopic techniques. Different *Trebouxia* spp. were detected as primary ~~phycobiont~~photobiont in each island (*Trebouxia cretacea*-Fuerteventura, *T. asymmetrica*-Lanzarote and *Trebouxia* sp. `*arnoldoi´*-Tenerife). Coexistence of various *Trebouxia* spp. within a thallus were detected by using specific primers-PCR. Those three ~~phycobiont~~photobionts were isolated and cultured under laboratory conditions. ~~To relate the temperature with the presence of a specific *Trebouxia* species,~~ Different phytohormone profiles were obtained in the isolated strains which suggest different internal signalling needs. In addition, we characterized the~~ir~~ response of the isolated strains to different temperatures ~~were monitored~~ using chlorophyll fluorescence. *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In contrast, *Trebouxia* sp. *`arnoldoi´* and *T. cretacea* were more sensitive to changes in growing temperature decreasing Fv/Fm at 17º C. ~~Phytohormone profiles of isolated phycobionts were performed to delve deeper into the ecologically adapted phycobiont hypothesis~~. Our results indicate that *B. zoharyi* is flexible regarding the ~~phycobiont~~photobiont choice~~,~~ depending on the region ~~or the environmental conditions~~, and suggest ~~clearly influenced by~~that bioclimatic factors could influence the myco/photobiont association patterns ~~the temperature~~. ~~The results point to habitat specific adaptations which lead to similar behaviour in phycobionts which are genetically different. The physiological adaptations of lichen phycobionts may substantially contribute to the stress resistance strategy and the colonization capacity.~~

Keywords: coexistence, isolation, microalgae, photosynthesis, symbiosis, ultrastructure

Declarations

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Funding Funding for field and laboratory work for this study was provided by the Ministerio de Economia y Competitividad (MINECO and FEDER, Spain) (CGL2016-79158-P) and Prometeo Excellence in Research Program (Generalitat Valenciana, Spain) (PROMETEOII/2013/021; PROMETEO/2017/039). Daniel Sheerin revised the English manuscript.

Competing interests The authors declare no competing interests

Availability of data and material GenBank

Code availability LSU rDNA: MT458607-MT458609; nrITS MT458610-MT458618

Authors' contributions PM, AM and SC conceived the study and designed the laboratory part of the study. SC, PM and AM carried out laboratory work. FG-B and JR-A analyzed microscopic images. AC and PM performed photosynthesis measurements. FM and PC performed phytohormone quantifications. AM and PM analyzed all data and wrote the manuscript. All authors edited and approved the final version of the manuscript.

ACKNOWLEDGMENTS

Dr. Arnoldo Santos ~~(Tenerife)~~ was involved in the design of the surveys and the sample collection, also in the ecology and bioclimatic information of the locations. Daniel Sheerin revised the English manuscript. We dedicate this article to Eva Barreno in honour of her retirement.

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Introduction

The Canarian Archipelago contains eight volcanically active islands proximal to the African continent and the High Atlas Mountains. The origin and magmatic evolution of these islands have been contentious issues for several decades and make this particular archipelago unique for biodiversity studies. The Canary Islands are famous for their extraordinary diversity in lichenized and lichenicolous fungi (Van den Boom and Etayo 2006; Hernández Padrón and Pérez Vargas 2010). The diversity of lichenized algae has only been studied partially for *Tephromela atra* (Muggia et al. 2010), *Ramalina farinacea* (Casano et al. 2011; del Campo et al. 2013; Moya et al. 2017), *Lecanora rupicola* (Blaha et al. 2006), *Parmotrema pseudotinctorum* (Molins et al. 2013; Škaloud et al. 2018), *Stereocaulon vesuvianum* (Vancurová et al. 2015), *Psora decipiens* (Moya et al. 2018) and *Cladonia* spp. (Moya et al. 2015).

Lichen symbioses are currently considered as microecosystems in which multispecies assemblages are hosted in the lichen thallus (holobiont), formed by the two major lichen symbionts, e.g. the mycobiont (fungal partner) and the photobiont (a population of photosynthetic green or blue-green algae). According to the most recent investigations, many other fungi (Muggia and Grube 2018; Smith et al. 2020), non-photosynthetic bacteria (e.g. Aschenbrenner et al. 2014; Grube et al. 2015; Cernava et al. 2017; Sierra et al. 2020) and photosynthetic green microalgae co-inhabit within the lichen thalli, giving rise to the peculiar phenotypes, and which may contribute to the diverse patterns of secondary metabolites (Spribille 2018) and environmental adaptation. In terms of lichen ~~phycobiont~~photobionts, intrathalline microalgal diversity, e.g. multiple ~~phycobiont~~photobiont species within a single lichen thallus, has previously been observed in a number of lichen symbioses (Dal Grande et al. 2014; Muggia et al. 2013; Moya et al. 2017; Škaloud et al. 2018). In some cases, algae with different physiological performances are ~~ever~~- present in lichen thalli, potentially facilitating the success of these lichens in a wide range of habitats and geographic areas and/or in changing environmental conditions (Casano et al. 2011; del Hoyo et al. 2011).

Muggia et al. (2020) highlight the need for an integrative taxonomic approach, incorporating morphological and physiological data from axenic cultures with genetic data, to establish a robust, comprehensive taxonomy for *Trebouxia*. Complementation with ecophysiological studies needs to be employed to explain how lichens thrive ~~adapt and outcompete~~ in extreme environments (Sadowsky and Ott 2012). Lichen ~~phycobiont~~photobionts show a particular photosynthesis performance and have evolved alternative photosynthetic-machinery protective mechanisms in response to their special ecophysiology (Gasulla et al. 2019). The photosynthesis responses to abiotic factors, like temperature and light conditions in axenic cultures of ~~phycobiont~~photobionts, reflects the ecophysiological plasticity of this symbiosis as a mechanism, allowing the lichen to adapt to changing and often stressful environments (Casano et al. 2011). ~~The study of biomass accumulation and photosynthetic traits, including the photochemical efficiency of photosystem II (PSII), the reduction-oxidative state of QA, and non-photochemical quenching (NPQ) denote which holobiont has improved its ecological fitness (Casano et al. 2011).~~

Phytohormones are chemical messengers involved in several physiological, stress response and biochemical processes of higher plants at very low concentrations. Phytohormone composition has been characterized in only a few algae (Yokoya et al. 2010; Gupta et al. 2011; Wang et al. 2014), but recently, Pichler et al. (2020) studied phytohormone composition in aeroterrestrial Trebouxiophyceae, including three lichen- forming microalgae. The results obtained in this study contribute to valuable baseline information for further studies into the roles of phytohormones in

microalgae. Including this technique to characterize isolated symbiotic microalgae could help to understand the ecologically adapted ~~phycobiont~~photobionts hypothesis.

Buellia zoharyi is a circum-Mediterranean/Macaronesian species forming crustose placodioid lichens that usually occur~~s~~ in biocrusts in semi-arid areas of the Mediterranean region (Gutiérrez-Carretero and Casares-Porcel 2011). Specifically, this species predominantly grows on gypsum soils (Crespo and Barreno 1975; Barreno 1994; Trinkaus and Mayrhofer 2000), but occasionally some interesting populations~~communities~~ of this species grow on basaltic lava flows in the Canary Islands (Etayo 2011; Giralt and Van den Boom 2011; Roux and Poumarat 2015). The presence of this lichen species in the Canary Islands was reported in Lanzarote (Trinkaus and Mayrhofer 2000), Fuerteventura (Van den Boom and Etayo 2006) and Tenerife (Chiva et al. 2019), and populations in the other four Islands must be scarce or even non-existent. ~~T~~These three locations have~~showed~~ similar ecological conditions e. g. the same high irradiance (5.5 – 5.7 KWh/m2) moreover, in the three locations *B. zorayi* grown forming ~~similar ecological conditions, such as~~ biocrusts in areas with sparse vascular vegetation ~~and high irradiance (5.5 – 5.7 KWh/m2)~~. However, the WordlClim database reported different mean annual temperatures (MAT) ~~in the three locations~~: 17º C in Fuerteventura and Lanzarote, and 20º C in Tenerife (https://www.worldclim.org; Hijmans et al. 2005). Chiva et al. (2019) analyzed mycobiont diversity in *B. zoharyi* in the Mediterranean region including those three Canary Island locations. They found low genetic diversity and two geographically differentiated haplogroups: one including populations from the Iberian Peninsula to Azerbaijan, and the other from the southern Iberian Peninsula, North Africa and the Canary Islands. Complementary analyses of *B. zoharyi* concerning the range of associated ~~phycobiont~~photobionts and their ecophysiological traits will be necessary to identifying historical and ecological factors at the basis of the evolutionary history of this group of soil- dwelling taxa.

Here we applied a multidisciplinary approach to describe~~epict~~ microalgae diversity from *B. zoharyi* ~~growing~~ settled in the Canary Islands. Populations included in this study cover the entire described range of distribution in the ~~Canary Islands~~ archipelago. ~~Phycobiont~~Photobionts were characterized in symbiosis ~~by means of~~using molecular and microscopic techniques. Coexistence of various *Trebouxia* spp. were detected by using specific primers-PCR. Representative ~~phycobiont~~photobionts were isolated and cultured under laboratory conditions. We ~~. They were~~ characterized the isolated strains ~~by means of~~using ~~-~~molecular, microscopy and spectrometry techniques. Their responses to different temperatures were monitored using chlorophyll fluorescence to answer if this bioclimatic factor could influence the myco/photobiont association patterns. ~~answer if temperature drives the~~

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~~presence of a specific *Trebouxia* species in each location, and whether or not the complementary physiological behaviour of each algal species, globally improves the ecological fitness of the holobiont under global warming temperature conditions.~~

Material and methods

Sampling and DNA extraction

In this study, 46 thalli of *Buellia zoharyi* collected from Lanzarote, Tenerife and Fuerteventura were analyzed (Table 1S). We included fresh (N=43) and herbarium samples (N=3). Fresh specimens were air-dried for one day after sampling and then stored at -20°C. Lichen thalli were examined under a stereomicroscope to remove soil particles and were immersed sequentially in ethanol and NaOCl (Arnold et al. 2009) to remove surface contaminants and to ensure the intrathalline origin of the sequenced microalgae. Fragments from different parts of each thallus were randomly excised and pooled together. Total genomic DNA was isolated and purified using the DNeasy Plant Mini kit (Qiagen, Hilden, 121 Germany) following the manufacturer's instructions.

Primary ~~phycobiont~~photobiont PCR amplification and Sanger sequencing

Two algal loci were amplified; a region of the chloroplast LSU rDNA gene using the algal specific primer pair 23SU1 and 23SU2 (del Campo et al. 2010a) and the nrITS (internal transcribed spacer) using the primer pair nr-SSU-1780 (Piercey-Normore and DePriest 2001) and ITS4 (White et al. 1990). PCR reactions were performed following Moya et al. (2018). The PCR products were visualized 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 ABI 3730XL using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Sanger sequences were visualized and manually evaluated with Chromas 2.6.6.0 (http://technelysium.com.au/wp/chromas/).

Trebouxia ~~phycobiont~~photobiont phylogenetic analysis

The nrITS and LSU rDNA datasets were collapsed using TCS 1.21 (Clement et al. 2002). The dataset included the newly determined nrITS and LSU rDNA sequences from thalli and isolated ~~phycobiont~~photobiont (accession numbers LSU rDNA: MT458607-MT458609; nrITS: MT458610-MT458618), and a selection of 30 *Trebouxia* species sequences available from the Culture Collection of Algae at Goettingen University (SAG), from the Culture Collection of Algae at the University of Texas (UTEX) and from the Symbiotic Microalgal Collection of the University of

Valencia (ASUV, *Trebouxia* sp. TR9 and *Trebouxia crespoana*). We included *Asterochloris mediterranea* as an outgroup.

A multiple alignment was built using MAFFT 7.0 (Katoh and Standley 2013) using default parameters. Aligned sequences were improved by eliminating the ambiguous regions using Gblocks 0.91b (Castresana 2000) with the least stringent parameters. This software allows conflicting regions in the alignment to be automatically removed. Both loci were concatenated, yielding an alignment of 1329 characters. The final matrix contained 42 ITS rDNA and 33 LSU rDNA sequences.

For each locus, the most appropriate substitution model was estimated using the Akaike information criterion (AIC) using JModelTest 2.1.4 (Darriba et al. 2012). The most appropriate nucleotide substitution models for nrITS and LSU rDNA were GTR+I+G and GTR+G, respectively. The phylogenetic trees of both loci were inferred by Bayesian inference (BI) and Maximum Likelihood (ML) approaches carried out on partitioned datasets using the different substitution models selected by JModelTest. ML analysis was implemented in RAxML 8 (Stamatakis 2014) using the GTRGAMMA substitution model. Bootstrap support (BS) was calculated based on 1,000 pseudoreplicates (Stamatakis et al. 2008). BI was carried out in MrBAYES 3.2 (Ronquist et al. 2012). Settings included two parallel runs with six chains over 20 million generations, starting with a random tree and sampling after every 200th step. We discarded the first 25% of the data as burn-in, and the corresponding posterior probabilities (PPs) were calculated from the remaining trees. Estimated sampled sized (EES) values above 200, and potential scale reduction factor (PSRF) values approaching 1,000, were considered indicators of chain convergence. The phylogenetic tree was visualized in FIGTREE 1.4.2 (Rambaut 2014; http://tree.bio.ed.ac.uk/software/figtree/). All analyses were run at the CIPRES Science Gateway 3.3 webportal (Miller et al. 2010).

Field Code Changed

Microscopic examinations “in thallus”.

The ultrastructure of the ~~phycobiont~~photobionts was characterized by transmission electron microscopy (TEM) from selected thalli (LA7, LA22, TE1, TE10 and FU1). For TEM, the cells were fixed and dehydrated as described in Molins et al. (2018a). Samples were embedded in Spurr’s resin according to the manufacturer’s instructions. Sections (90 nm) were cut and mounted as described in Moya et al. (2018). 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.

Secondary *Trebouxia* strains detected by specific PCR primers

To detect the presence of secondary *Trebouxia* species in each thallus, PCR were performed using as templates each PCR from the primary ~~phycobiont~~photobiont (obtained with the primer pair nr-SSU-1780 / ITS4) and re-amplifying with specific primer pairs (Table 2S). These specific forward and reverse primers were designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia cretacea* and *Trebouxia* sp. *`arnoldoi´*.

Isolation, PCR identification and propagation of microalgae strains.

~~Phycobiont~~Photobionts were isolated from selected thalli (LA7, TE1 and FU1) using two protocols: a) the micromethod described by Gasulla et al. (2010), where the resulting algal suspension was diluted with sterile water and spread using the 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 Muggia et al. (2014) where tiny clumps of the algal layer were inoculated directly into BBM. The isolated algae were maintained under a 50 µmol/ $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) with a 12 h photoperiod at 20°C. Subsequent subcultures were performed until we obtained a unialgal culture and fast PCR microalgae identification was performed directly from the colonies as described in Molins et al. (2018b).

Microscopic investigations of ~~phycobiont~~photobionts "in culture".

Light microscopy (LM) and Epifluorescent Microscopy (EFM) was performed on selected unialgal cultures on the 21st day of cultivation at 20º C. All LM and EFM observations were carried out with an Olympus Provis AX 70 fluorescence microscope equipped with an Infinity 2–3 C Lumenera® digital camera and analyzed with "Infinity Analyze" Software. For EFM, an Olympus U-ULS 100 HG epifluorescence system with U-MWBV (excitation filter 400–440 nm, dichroic mirror 455 nm, barrier filter 475 nm) cubes was used.

Phytohormone content determination

Endogenous phytohormone levels of 21-day-old microalgae cultures grown at 20º C were determined according to the protocol of Durgbanshi et al. (2005). Lyophylized microalgae samples were processed by extraction in 5 ml of distilled water after fortifying them with internal standards: [$^2H_6$] - abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)), dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation according to Kristl et al. (2005)), [$^2H_2$]-indole-3-acetic acid (IAA) (10 ng, Sigma-Aldrich) and [$^2H_4$] -salycilic acid (SA) (100 ng, Sigma-Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0 using a 30% (v / v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of ethyl ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a clean vial, combining both partitions and drying them under vacuum using an evaporation centrifuge coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint-Herblain Cedex, France). The dried residue was resuspended by adding 100 µL of methanol (HPLC grade, Scharlau) in the test tube by ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure water

(MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore diameter of 0.2 µm before analysis. Phytohormone analysis was performed using an HPLC device (Alliance 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an electrospray interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and separated by a reverse phase column (Kromasil 100, C18, 5 µm, 100 × 2.0 mm, Scharlau) using a linear gradient of methanol and ultrapure water, supplemented with acetic acid to a final concentration of 0.01% (v /v) and a flow of 0.3 mL / min. Discrimination and detection of each analyte was carried out following the fragmentation pattern and the characteristic retention time. The ionization and collision conditions for each compound were optimized by direct infusion of pure standards (approximately 5 mg/L). The quantification of the analytes of interest was performed using the response factor (analytical area/area) by interpolation in a calibration curve injected alternatively in the samples. Chromatogram processing, integration and quantification were performed using MassLynx 4.0 software. The profiles of the relative content of phytohormones between strains were determined with the Heatmapper web tool (Babicki et al. 2016).

Measurement of chlorophyll fluorescence imaging from isolated algae

Fast PCR was performed directly from the unialgal cultures as described in Molins et al. (2018b) to ensure the purity and identity of the selected aliquot used for chlorophyll (Chl) fluorescence images (CFI) measurements.

A 50 ml aliquot of these actively growing algae, resuspended in liquid BBM medium, was inoculated on cellulose–acetate disks placed on agarized BBM medium and then cultured for 21 days under two conditions, 17°C and 20°C with a 50 µmol $m^{-2}s^{-1}$ PPFD under a 12 h/12 h light/dark cycle.

CFI was performed using an imaging-PAM fluorometer (Walz, Effeltrich, Germany), in order to investigate the behavior of Chl fluorescence parameters in the three algae at different light intensities and grown under different temperature conditions (17°C and 20°C). Algae samples were layered on filter paper that was kept moist with distilled water in order to maintain the cells in a fully hydrated state. The algae membranes were darkened for 30 min prior to measurement. Chl *a* fluorescence determinations were obtained from n= 4 samples for each algale strain and temperature. ~~Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and the other in the outer zones in order to evaluate spatial heterogeneity~~. The minimum (dark) fluorescence (F_o) was obtained by applying measuring light pulses at a low frequency (1 Hz). The maximum fluorescence (F_m) was determined by applying a saturating blue

pulse (10 Hz). The maximum quantum yield of PSII photochemistry (Rohácek 2002), often called the F_v/F_m ratio, was determined as $F_m - F_o/F_m$. Then, a light curve with actinic illumination from 0 to 964 µmol photons m^{-2} s^{-1} was switched on, and saturating pulses were applied for 20 s in order to determine the maximum fluorescence yield (F'_m) intensity, and the steady-state fluorescence value that is immediately prior to the saturating pulse is F_s. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation NPQ = $(F_m - F'_m)/F_m$ (Bilger and Björkman 1991). The actual quantum efficiency of PSII photochemistry, Φ_{PSII} , was calculated according to Genty et al. (1989) by the formula: $(F'_m - F_s)/F'_m$), and the coefficient of photochemical quenching (Van Kooten and Snel 1990) was determined as qP = $(F'_m - F_s)/(F'_m - F'_o)$. Excitation pressure on PSII (QA), which reflects the proportion of the primary quinone electron acceptor of PSII that is in the reduced state, was calculated as 1 − qP (Demmig-Adams et al. 1996). The relative electron transport rate (ETR) was calculated as $\Phi_{PSII} \times PAR \times 0.84 \times 0.5$ (Schreiber et al. 1986). To determine F'_o correctly, it would be necessary to switch off the actinic light and quickly reoxidise the PSII acceptor side with the help of far-red light, but this is not feasible with imaging-PAM as far-red light would penetrate the CCD-detector and cause serious disturbances to fluorescence images (see http://www.walz.com). The value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m + F_o/F'_m)$. For each interval, saturation pulse images and values of various Chl fluorescence parameters were captured (Calatayud et al. 2006).

Two AOI were selected one in the central part and other in the outer algae zones in order to evaluate spatial heterogeneity CFI parameters between both AOI. After comparing both AOI for each algae strain and temperature, not differences were observed for any Chl fluorescence parameters (data not shown). Then, the values of Chl fluorescence parameters displayed in the figures (4a and 5) are means of both AOI.

~~**Phytohormone content determination**~~

~~Endogenous phytohormone levels of 21-day-old microalgae cultures grown at 20º C were determined according to the protocol of Durgbanshi et al. (2005). Lyophylized microalgae samples were processed by extraction in 5 ml of distilled water after fortifying them with internal standards: [2H6]- abscisic acid (ABA) (100 ng, prepared as described in Gómez-Cadenas et al. (2002)), dihydrojasmonic acid (JA) (100 ng, synthesized in the laboratory by catalytic hydrogenation according to Kristl et al. (2005)), [2H2] indole-3-acetic acid (IAA) (10 ng, Sigma-Aldrich) and [2H4]- salycilic acid (SA) (100 ng, Sigma-Aldrich). The extracts were centrifuged at 4000 x g, at 4° C for 45 min. Subsequently, the supernatant was collected in clean tubes and the pH adjusted to 3.0~~

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~~using a 30% (v / v) acetic acid solution. The acidified extracts were partitioned twice with 3 ml of ethyl ether (ACS grade, Scharlau, Barcelona, Spain). The upper organic phase was recovered in a clean vial, combining both partitions and drying them under vacuum using an evaporation centrifuge coupled to a cold trap (RC 10.22 and RT 10.90, Jouan, Saint Herblain Cedex, France). The dried residue was resuspended by adding 100 µL of methanol (grau HPLC, Scharlau) in the test tube by ultrasound for 10 min. Subsequently, the final volume of 1 mL was completed with pure water (MiliQ). The resulting solution was filtered using regenerated cellulose filters with a pore diameter of 0.2 µm before analysis. Phytohormone analysis was performed using an HPLC device (Alliance 2860, Waters Corp., Milford, USA) coupled to a tandem mass spectrometer with an electrospray interface (Quattro LC, Micromass, Manchester, UK). The samples were injected and separated by a reverse phase column (Kromasil 100, C18, 5 µm, 100 × 2.0 mm, Scharlau) using a linear gradient of methanol and ultrapure water, supplemented with acetic acid to a final concentration of 0.01% (v /v) and a flow of 0.3 mL / min. Discrimination and detection of each analyte was carried out following the fragmentation pattern and the characteristic retention time. The ionization and collision conditions for each compound were optimized by direct infusion of pure standards (approximately 5 mg/L). The quantification of the analytes of interest was performed using the response factor (analytical area/area) by interpolation in a calibration curve injected alternatively in the samples. Chromatogram processing, integration and quantification were performed using MassLynx 4.0 software. The profiles of the relative content of phytohormones between strains were determined with Heatmapper web tool (Babicki et al. 2016).~~

~~**Statistical analyses**~~

~~Data was analysed by one-way Analysis of Variance (ANOVA) followed by post-hoc comparisons by Tukey's HSD test. A probability value < 0.05 was considered statistically significant. Calculations were performed using R Software.~~

Statistical analyses

Data was analysed by one-way Analysis of Variance (ANOVA) followed by post-hoc comparisons by Tukey's HSD test. A probability value < 0.05 was considered statistically significant. Calculations were performed using R Software.

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Results

Primary *Trebouxia* diversity detected by Sanger sequencing (nrITS)

The concatenated aligned algal nrITS + LSU rDNA fragment was 1329 bp in length. BI and ML phylogenetic hypotheses were topologically congruent. In the 46 samples, we detected three *Trebouxia* species (Fig. 1). According to the clade code introduced for *Trebouxia* by Leavitt et al. (2015), and subsequently applied by Moya et al. (2017) and Muggia et al. (2020), these *Trebouxia* species belong to clade 'A' *arboricola/gigantea* type, precisely: *Trebouxia asymmetrica* in thalli from Lanzarote, *Trebouxia cretacea* in Fuerteventura, and *Trebouxia* sp. `*arnoldoi´* in Tenerife.

Ultrastructural characterization of *Trebouxia*

Transmission electron microscopy (TEM) analyses of ~~phycobiont~~photobionts based on the ultrastructure of pyrenoids (Py) and plastids (Chl) distinguished at least three different *Trebouxia* morphotypes. Morphological characteristics of each morphotype, in detail, can be seen in Fig. 2 and Fig. 1S.

One morphotype was found in *Buellia zoharyi* cells from Lanzarote (Fig. 2a; b). They showed a single central Py related to the gigantea type described by Friedl (1989) with pyrenoglobuli (Pg) uniformly distributed within the Py matrix. Lobulated-stellate chloroplast with abundant lax thylakoid membranes forming stacks of 4-5 membranes (grana).

The second morphotype was found in *B. zoharyi* cells from Fuerteventura (Fig. 2c, d). These cells presented a single central Py related to the impressa/gigantea type described by Friedl (1989), with a highly lobulated chloroplast with abundant dense thylakoid membranes forming stacks of 2-3 membranes.

The third morphotype was detected in *B. zoharyi* cells from Tenerife (Fig. 2e, f). They presented a single central Py related to the impressa/gigantea type described by Friedl (1989). The chloroplast morphology was similar to the second morphotype (lobulated with dense abundant thylakoid membranes forming stacks of 2-3 membranes). Large peripheral vesicles surrounding the chloroplast were present.

***Trebouxia* multiplicity revealed by specific PCR primers**

Herbarium samples were excluded from this analysis. Specific forward and reverse primers were designed in this study based on the nrITS sequences obtained with the primer pair nr-SSU-1780 / ITS4 in *B. zoharyi* from the Canary Islands, which included *Trebouxia asymmetrica*, *Trebouxia cretacea* and *Trebouxia* sp. `*arnoldoi´*

All the samples from Lanzarote (n=23) showed *T. asymmetrica* as the primary ~~phycobiont~~photobiont, and specific PCR detected *T. cretacea* in 22 thalli. *Trebouxia* sp. `*arnoldoi´* was not detected (Fig. 3; Table 3S). Samples from Fuerteventura (n=7) showed *T. cretacea* as the primary ~~phycobiont~~photobiont. The presence of secondary algae was detected only in three thalli: two of them revealed *T. asymmetrica* as the secondary ~~phycobiont~~photobiont and one *Trebouxia* sp. `*arnoldoi´* (Fig. 3; Table 3S). Samples from Tenerife (n=13) had *Trebouxia* sp. `*arnoldoi´* as the primary ~~phycobiont~~photobiont. All the samples only showed *T. asymmetrica* as the secondary ~~phycobiont~~photobiont. *T. cretacea* was not detected (Fig. 3; Table 3S).

Identification and morphological characterization of isolated *Trebouxia* spp. from *Buellia zoharyi*

To corroborate the purity and identity of the unialgal selected aliquot, fast PCR was performed directly from the cultures as described in Molins et al. (2018b). Sequences obtained were included in the phylogeny indicated as isolated ~~phycobiont~~photobiont (Fig. 1). We detected three *Trebouxia* spp.*: T. asymmetrica, T. cretacea* and *Trebouxia* sp. *`arnoldoi´*.

All three *Trebouxia* spp. presented mature vegetative cells, mostly unicellular and spherical as seen using LM and EFM (Fig. 2S). Tetrads and octads were only observed in *T. cretacea*. The spherical vegetative cells were 14–16 (19) µm in diameter. All cells showed the characteristic *Trebouxia* central chloroplast dissected into lobes (Fig. 2S).

Phytohormone profiles of *Buellia zoharyi* photobionts

Phytohormone endogenous content was also determined to characterize *Trebouxia* strains cultured in BMM at 20º C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA, JA conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me-JA) (Fig. 4). The *Trebouxia* strains isolated from *B. zoharyi* showed differences in their phytohormone profiles:

1) *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 4a, b), *Trebouxia* sp. *`arnoldoi´* showed a different profile, with about double the ABA endogenous content compared to the other strains (Fig. 4b), and higher levels of IAA (about five times) than *T. asymmetrica* and *T. cretacea* (Fig. 4a).

2) SA and JA-Ile levels also showed a different profile between microalgae strains, with similar levels for *T. cretacea* and *Trebouxia* sp. *`arnoldoi´*, while *T. asymmetrica* showed

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the highest level of SA (6 times greater; Fig. 4c) and higher levels of JA-Ile than *T. cretacea* (about 400% more; Fig. 4d).

3) Me-JA content of the *Trebouxia* strains showed another different profile, with similar levels for *T. asymmetrica* and *Trebouxia* sp. `*arnoldoi*´, which were about 2.6 times higher than the levels observed for *T. cretacea* (Fig. 4d).

Chlorophyll *a* fluorescence~~Photosynthesis~~ response to different temperatures (20º C and 17º C) ~~and phytohormone profiles~~ of *Buellia zoharyi* ~~phycobiont~~photobionts

According to the F_v/F_m measurements (Fig. 5~~4~~a), *Trebouxia* sp. `*arnoldoi*´ was significantly affected ~~significantly~~ by temperature, with the lowest F_v/F_m values at 17º C. Similarly, i~~I~~n *T. cretacea*, F_v/F_m showed a significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17º C However, *Trebouxia asymmetrica* showed similar values at 20º C and 17º C (0.66 ± 0.02 and 0.62 ± 0.05), respectively, without significant differences between them~~).~~ ~~In *T. cretacea*, F_v/F_m showed a significant decrease from 0.65 ± 0.02 to 0.55 ± 0.03 with a lower value at 17º C~~. The highest F_v/F_m values were obtained for *T. asymmetrica* and *T. cretacea* at 20º C.

~~The mean of images pixels from CFI for F_v/F_m inside the two areas did not display significant differences between internal and external areas (data not show).~~ The observation of color changes, ranging from black (0.000) to pink (1.000), revealed that the images for F_v/F_m showed a uniform color for algae discs, and that different intensities of blue colors associated with higher values of Fv/Fm (Fig. 4b) for *T. asymmetrica* and *T. cretacea* at 20º C, and with the lowest F_v/F_m values (green) for *Trebouxia* sp. `arnoldoi´ at 17º C (Fig. 5~~4~~b).

All NPQ values increased with respect to light intensity, but with a different shape and magnitude ~~were observed~~ depending on the *Trebouxia* species and growth temperature (Fig. 6~~5~~a)~~.~~; ~~however, I~~in the case of *Trebouxia* sp. `*arnoldoi*´ and *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes showing the same kinetic shape, but *Trebouxia* sp. `*arnoldoi*´ showed the highest NPQ values ~~the NPQ value increased steeply at higher light intensities at both temperatures showing the same kinetic shape~~. ~~*T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes.~~ ~~For~~, *T. cretacea* at 20º C and *T. asymmetrica* displayed similar NPQ values and at the end of light-curve kinetics *T. cretacea* showed the highest NPQ values.

~~*T. cretacea*, the highest NPQ values at the end of light curve kinetic were for the algae grown at 20º C. Nevertheless, *T. asymmetrica* NPQ exhibited similar values for both temperature growth regimes.~~

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Regarding ETR (Fig. 6~~5~~b), their values were slightly higher for *Trebouxia* strains growing at 20 ~~º~~ ª C compared with 17º C. Among species, *T. cretacea* displayed the highest ETR values, mainly at the end of light curve kinetic at 20º C and the minimum values were observed in *Trebouxia* sp. *`arnoldoi´* at 17º C.

~~.~~

Photosystem II excitation pressure, expressed as 1-qP (photochemical quenching; Fig. 6~~5~~c), increased gradually with light intensity in the three algal strains~~., with~~ In general, *T. asymmetrica* ~~at 20º C~~ and *T. cretacea* showed~~ing~~ ~~slightly higher~~similar values, however ~~-~~*Trebouxia* sp. *`arnoldoi´*showed slightly values except at high irradiance where ~~compared to *Trebouxia* sp. *`arnoldoi´* at 20º C.~~ *Trebouxia* sp. *`arnoldoi´* at 17º C showed the highest values ~~at high irradiance~~.

~~Phytohormone endogenous content was also determined for *Trebouxia* strains cultured in BMM at 20º C for 21 days. Mass spectrometry analysis showed detectable levels of IAA, ABA, JA conjugated to the amino acid Isoleucine (JA-Ile) and methyl jasmonate (Me-JA) (Fig. 6). The *Trebouxia* strains isolated from *B. zoharyi* showed some differences in their phytohormone profiles. While *T. asymmetrica* and *T. cretacea* presented similar levels of IAA and ABA (Fig. 6a, b), *Trebouxia* sp. *`arnoldoi´* showed a different profile, with about double the ABA endogenous content compared to the other strains (Fig. 6b), and higher levels of IAA (about five times) than *T. asymmetrica* and *T. cretacea* (Fig. 6a). SA and JA-Ile levels showed a different profile between microalgae strains, with similar levels for *T. cretacea* and *Trebouxia* sp. *`arnoldoi´*, while *T. asymmetrica* showed the highest level of SA (6 times greater; Fig. 3c) and higher levels of JA-Ile than *T. cretacea* (about 400% more; Fig.6d). Finally, Me-JA content of the *Trebouxia* strains showed another different profile, with similar levels for *T. asymmetrica* and *Trebouxia* sp. *`arnoldoi´*, which were about 2.6 times higher than the levels observed for *T. cretacea* (Fig. 6d).~~

Discussion

This study applied a multidisciplinary approach to describe~~pict~~ microalgae diversity from *B. zoharyi* growing in the Canary Islands. Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the region, and suggest that bioclimatic factors could influence the myco/photobiont association patterns. ~~Phycobionts were characterized in symbiosis by means of molecular and microscopic techniques. Representative phycobionts were isolated and cultured, and were characterized by different procedures. Their response to different temperatures were monitored~~

~~using chlorophyll fluorescence to discover if temperature drives the presence of a specific *Trebouxia* species in each location, and if the complementary physiological behaviour of each algal species explains how lichens adapt and outcompete in different environments.~~

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According to Beck et al. (2002), 'selectivity' in lichens refers to the taxonomic range of partners that are selected by one of the bionts, while 'specificity' should be used for the symbiotic association, and depends on the range and taxonomic relatedness of acceptable partners. Lichens with high selectivity may associate with a limited number of ~~phycobiont~~photobionts. Numerous lichen-forming fungi~~mycobionts~~ have been shown to associate with identical species of *Trebouxia*, while others exhibited higher ~~phycobiont~~photobiont flexibility (Kroken and Taylor 2000; Ohmura et al. 2006, 2018; Doering and Piercey-Normore 2009; Leavitt et al. 2013, 2015; Lindgren et al. 2014). Our results indicate that *B. zoharyi* are flexible in their ~~phycobiont~~photobiont choice, as they associate with three *Trebouxia* species in 300 km (distance from Igueste de San Andrés; Tenerife to Los Valles; Lanzarote) ~~in a delimited geographic area (approx. 1000 km)~~.

~~Phycobiont~~Photobiont diversity can be shaped by the reproductive and dispersal strategies of the mycobiont (Cao et al. 2015, Steinová et al. 2019), geography (Muggia et al. 2014, Werth and Sork 2014, Leavitt et al. 2015), growth substrate (Bačkor et al. 2010, Leavitt et al. 2013, Muggia et al. 2014) and macroclimate (Lu et al. 2018, Singh et al. 2018). ~~An important mechanism controlling symbiotic associations may be the symbiont's reproductive mode~~. Lichens that reproduce sexually via independent dispersal of fungal spores, undergo a process of re-lichenisation. This means that the germinating spore of the mycobiont can easily exchange its autotrophic partner, in contrast to asexually reproducing lichens distributing both partners together, which allows the continuation of the symbiosis without the need to re-associate with another biont (Beck et al. 1998, 2002; Romeike et al. 2002; Sanders and Lücking 2002). Lichens that depend on the cyclical establishment of fungal-~~phycobiont~~photobiont associations to colonize varied wide-ranging habitats might require a relatively higher flexibility in the specificity and ecological selection of their ~~phycobiont~~photobionts. This flexibility would facilitate successful re-lichenizations by allowing for alternative partnerships in each habitat (Romeike et al. 2002~~). However, some exceptions to this rule have been described; even asexually reproducing lichens, such as the *Lepraria* species, have been shown to switch their algal partners (Nelsen and Gargas 2008). In populations of *Physconia grisea* with a vegetative propagation strategy, mycobionts associate with more than one phycobiont genotype (Wornik and Grube 2010). It was also reported that both sexual and vegetative reproduction allows lichens to generate almost the same amount of diversity to adapt to their environments (Cao et al. 2015). Moreover, *Protoparmeliopsis muralis*, which does not produce~~

~~vegetative propagules, exhibited a low selectivity level (Guzow-Krzemińska 2006; Muggia et al. 2013).~~

The dispersal of *B. zoharyi* over medium to long distances can be accomplished by either meiotic ascospores produced in fertile thalli, or thallus fragments detached from fertile and sterile ones (Barreno 1994; Casares and Llimona 1983). Both ~~types of reproductive strategy~~strategies are suitable for long- distance dispersal, even across the Mediterranean Sea, based on evidence of other lichens showing widely disjunct populations (Alors et al. 2017; Fernández- Mendoza and Printzen 2013; Garrido- Benavent et al. 2018). Moreover, increased connectivity among the Canary Islands, Africa and the Iberian Peninsula possibly occurred during Pleistocene glaciations, when the distance between the Eastern most island (Fuerteventura) and the Moroccan coast was reduced to 60 km (Fernández- Palacios and Whittaker 2008). Chiva et al. (2016) showed *T. cretacea* as the predominant ~~phycobiont~~photobiont in 117 thalli of *B. zoharyi* from the Iberian Peninsula and Morocco. The colonization of the Canary Islands by *B. zoharyi* must have originated from the Moroccan coast to Fuerteventura (20.7 Ma), currently the eastern island, only 100 km from the African coast, evidenced as the maintenance of the symbiont pattern (*T. cretacea*). Carracedo (1994), dated the origin of Lanzarote in 15.5 Ma and Tenerife in 11.6 Ma. *B. zoharyi* must have colonized the Canary Islands subsequently and adopted ecologically adapted ~~phycobiont~~photobionts (*T. asymmetrica* and *Trebouxia* sp. `*arnoldoi´*) in those Islands.

The diversity of ~~phycobiont~~photobionts has only recently been explored by environmental DNA metabarcoding approaches, and has focused on species within the Mediterranean basin to date (Moya et al. 2017; Dal Grande et al. 2018; Smith et al. 2019). In contrast to high-throughput sequencing approaches, traditional and largely applied DNA barcoding using Sanger sequencing was able to detect only the principal ~~phycobiont~~photobiont in the thalli (Paul et al. 2018; Moya et al. 2020). In this study, the authors determined if the second most abundant microalga exceeded 30% of the total HTS reads in a sample, Sanger sequencing generally failed and generated ambiguous Sanger sequences showing double peaks. Moreover, in the present study no samples with double peaks were found in the electropherogram, the co-occurrence of multiple *Trebouxia* inside a thallus was performed by using specific primers. This approach may limit the detection of further associated algae due to specificity and thus, it should be used as a complement to traditional Sanger sequencing when it is not possible to perform HTS approaches. However, all analyses performed using the multi-copy nrITS showed methodological limitations that potentially bias the results presented, due to the variation in the copy numbers across microalgae species. Therefore, the relative abundance of algal groups inferred in this study with specific primers does not accurately describe~~pict~~ the true relative abundance of lichen-associated algae, given the potential for a very

wide range of nrDNA copy numbers of these algal groups. Even with these limitations, specific PCR primers revealed the presence of other secondary ~~phycobiont~~photobionts which would be available in the substrate. The presence of multiple algal species, and the different dominance of one of them in each species, implies the selection for a particular algal species by the mycobiont (Peksa and Škaloud 2011; Dupont et al. 2016). To corroborate this hypothesis, a complementary HTS approach, both from the lichen and from the substrate, should be included.

A reliable definition of ~~phycobiont~~photobiont species requires the description of morpho-anatomical traits both axenically cultured and in symbiosis. However, such traits are absent for the majority of lineages described in molecular phylogenetic analyses (Muggia et al. 2020). In this study, ~~phycobiont~~photobionts from Lanzarote, Fuerteventura and Tenerife were characterized in *Buellia zoharyi* thalli using transmission electron microscopy (TEM). ~~Phycobionts were also visualized from unialgal cultures using light microscopy (LM) and epifluorescent microscopy (EFM). To delimit algal species, LM and EFM techniques were less resolutive than TEM~~. ~~In this study,~~ The t~~t~~hree morphotypes ~~were~~ characterized structurally ~~by means of transmission electron microscopy~~, correspond~~ed~~~~ing~~ to each *Trebouxia* species molecularly described for each Island. These three *Trebouxia* species belong to clade 'A' *arboricola/gigantea* (Fig. 1) and showed, by TEM, a similar pyrenoid type (Fig. 2 and Fig. 1S), corresponding to the *impressa/gigantea* type described by Friedl (1989). Although Lanzarote´s morphotype showed a high similarity to the *gigantea* type, this trait does not allow us to differentiate each morphotype. In contrast to the pyrenoid, the chloroplast thylakoid arrangements clearly differentiated the three morphotypes. This coincides with further studies (Molins et al. 2018a) and evidences that the thylakoid arrangement is a key complement to the pyrenoid structure to characterize *Trebouxia* species. Current knowledge concerning *Trebouxia* phylogenetic relationships highlights the pressing needs to revise the original classification of the group proposed by Friedl (1989) as that classification does not match with Muggia et al.'s (2020) clade delimitation. The combination of molecular analyses together with ultrastructural techniques should be initiated to clarify taxonomic concepts to delimit new taxa of microalgae, and particularly in the case of *Trebouxia* diversity (Muggia et al. 2016, 2020; Moya et al. 2017; Molins et al. 2018b). Here we detected the new *Trebouxia* sp. *'arnoldoi'* which did not match any previously described *Trebouxia* spp. This provisional name has been proposed until it can be formally described: ~~"arnoldoi"~~ `arnoldoi´refers to the Canarian botanist Arnoldo Santos.

~~Incorporating morphological and ultrastructural traits from axenic cultures of candidate species circumscribed using molecular sequence data will be fundamental to facilitate a robust taxonomy within an integrative framework (Škaloud et al. 2018). Thus, I~~isolation procedures should be

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included in ~~phycobiont~~photobiont diversity studies~~, to properly concatenate molecular markers and~~ to perform morphological and physiological ~~studies~~analyses. In this study, the *Trebouxia* strains isolated from *B. zoharyi* were visualized from unialgal cultures using light microscopy (LM) and epifluorescent microscopy (EFM) and its phytohormone composition has been included to characterize them.~~-~~ This technique has been recently performed by Pichler et al. (2020) with the lichen forming algae *Trebouxia* sp., *Trebouxia decolorans* and *Asterochloris glomerata*. Under controlled experimental conditions, *T. cretacea*, *T. asymmetrica* and *Trebouxia* sp. `*arnoldoi*´ showed characteristic profiles of endogenous content in phytohormones (Fig. 4). IAA levels found in *Trebouxia* sp.*'arnoldoi'* (Fig. 4a) are similar to those observed in *Trebouxia* sp., *T. decolorans* and *A. glomerata* grown in solid media (Pichler et al. 2020). ABA production has been also detected in in these three microalgae (Pichler et al. 2020) as well as in *Trebouxia* sp. TR9 (Hinojosa-Vidal et al. 2018) isolated from *Ramalina farinacea* (Pichler et al. 2020). The ABA levels observed in the *Trebouxia* strains tested in this study (Fig. 4b) are in the same range of magnitude as the levels in *Trebouxia* sp. TR9, *Trebouxia* sp. and *T. decolorans* (Hinojosa-Vidal et al. 2018; Pichler et al. 2020). In contrast, the endogenous bioactive form of JA, JA-Ile (Fonseca et al. 2009), was found in all three *Trebouxia* strains from *B. zoharyi* (Fig. 4d), while no detectable endogenous levels of JA have been found by Pichler et al. 2020.)In addition, SA and Me-JA were also detected in the *Trebouxia* strains isolated in this study but they were not previously described in lichen-forming algae, although Me-JA has been detected in *Chorella* (Ueda et al. 1991).

In conclusion, the different phytohormone profiles obtained in the three *Trebouxia* strains isolated in this study (Fig. 4f), and in the other lichen forming algae mentioned above, suggest that each microalgae strain could present different internal signalling needs. On the other hand, it cannot be ruled out that a part of the production of these phytohormones could also be secreted to the external environment and play a role in external signalling mechanisms with the other members of the thallus symbiotic system. Related to this, the extracellular release of IAA, ABA and JA in the lichen-forming algae *Trebouxia* sp., *T. decolorans* and *Asterochloris glomerata*, has recently been described (Pichler et al. 2020). The determination of the role of these phytohormones in the internal signalling mechanisms of the photobionts, as well as in the coordination mechanisms with the rest of the elements of the lichen symbiosis, is a question that needs future studies.

~~In this study, further isolation, cultivation and physiological studies of three different *Trebouxia* species, should shed light on the ecological plasticity of the entire holobiont.~~ The worldwide distribution of lichen species was also hypothesized to be strongly correlated with the ecological specialization and the physiological performances of the ~~phycobiont~~photobionts (Casano et al.

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2011; Peksa and Škaloud 2011). In lichens, different degrees of mycobiont-~~phycobiont~~photobiont specificity is known (Leavitt et al. 2015; Beck et al. 2019; Steinová et al. 2019) and in some cases these relationships have been correlated to the suitability and the physiological performance of the ~~phycobiont~~photobionts in diverse, or even changeable, environmental settings (Vančurová et al. 2015; Steinová et al. 2019).

The lichen *Ramalina farinacea* (L.) Ach. has proved to be a suitable reference and model species for studying microalgal diversity, as it recurrently shows the co-occurrence of at least two ~~phycobiont~~photobionts (*Trebouxia* sp. TR9 and *T. jamesii*) inside the thalli using microscopy techniques, culture isolations and molecular characterization with different genetic markers (del Campo et al. 2010b, 2013). Moreover, the predominant ~~phycobiont~~photobiont differs between different populations; *T. jamesii* being the most abundant in the Iberian Peninsula and *Trebouxia*. sp. TR9 in the Canary Islands. Several studies have further demonstrated that these two ~~phycobiont~~photobionts respond differently to abiotic stresses (Casano et al. 2011). ~~The variety of ecological contexts in which *R. farinacea* proliferates, reflects the ecophysiological plasticity of this symbiosis as a mechanism allowing the lichen to cope and thus to adapt to changing and often stressful environments. (Casano et al. 2011; del Hoyo et al. 2011). Specifically,~~ Casano et al. (2011) also analysed the effects of temperature and light on the growth and the photosynthetic traits of isolated ~~phycobiont~~photobionts from *R. farinacea* (*Trebouxia* sp. TR9 and *T. jamesi~~s~~i*). They found that both species grew better at the lowest temperature and performed the experiments only in cultures grown at 17º C. On the contrary, we did not find such differences in the growth temperature, and thus, we performed the experiments at both 17º C (mean annual temperature in Fuerteventura and Lanzarote) and 20º C (mean annual temperature in Tenerife) to study the photosynthesis response to different temperatures (20º C and 17º C)~~if temperature drives the presence of a specific *Trebouxia* species in each location.~~

~~Chlorophyll fluorescence images in algae strains had allowed the study of the spatial heterogeneities of Chl fluorescence signatures over the whole area which cannot be detected through the conventional point measurements by non-imaging Chl fluorescence (Gorbe and Calatayud 2012). One of the most common parameter used to evaluate the physiological fitness of vegetable samples by fluorescence is the maximum quantum yield of PSII photochemistry, measured in the dark-adapted state, F_v/F_m. F_v/F_m images from lichen algae displayed homogeneity values; however, heterogeneity was shown in e.g. *Usnea antarctica* thallus associated with anatomy and its growth (Barták et al. 2004).~~

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The maximum quantum yield of primary photochemistry in dark-adapted leaves (F_v/F_m) in healthy plants reach F_v/F_m values around 0.840, but for lichens this parameter ranges between 0.550 and 0.700 (Casano et al. 2011; Gasulla et al. 2019). In this study F_v/F_m values ranged between 0.51-0.66. The decline in F_v/F_m might be a result of an increase in non-photochemical processes in the light harvesting antennae of PSII associated with a photochemical quenching down-regulation, photodamage of PSII reaction centres, or both (Osmond et al. 1993). In this study, we observed that *T. asymmetrica* did not modify their F_v/F_m values with respect to temperature acclimation. In contrast, *Trebouxia* sp. ~~*"arnoldoi"*~~ *`arnoldoi´* and *T. cretacea* were more sensitive to changes in growing temperature, showing a significant decline in F_v/F_m when grown at 17º C with respect to 20º C~~, which could indicate sensitivity to photoinhibition~~. In addition, these species showed the lowest ETR, mainly from 400µmol photons m^{-2} s^{-1} to the end of light curve. The electron transport rate was saturated at approximately the same irradiance (700 µmol photons m^{-2} s^{-1}) in all *Trebouxia* species (except *T. cretacea*) under both temperatures; similar results were obtained in *Trebouxia* sp. TR9 and *T. jamesii* (Casano et al. 2011). A decrease in ETR and an increase in 1-qP indicate a reduction in the quantum yield of PSII and a reduction state of the first electron acceptor of PSII, QA, respectively. In this circumstance, non-photochemical processes (NPQ) must be increased to guarantee excitation energy dissipation (Havaux et al. 1991) such as occurred in *Trebouxia* sp. *`arnoldoi´* where higher NPQ and 1-qP, and the lowest ETR, was observed. NPQ plays an important role in plants against excess radiation. In these algae strains from *Buellia zoharyi*, NPQ increases in all of them reducing the excitation pressure on reaction centres, thereby decreasing the possibility of photodamage (Papageorgiou and Govindjee 2014). These different behaviours between algae strains grown under two temperatures have been observed by Casano et al. (2011) in *Trebouxia* sp. TR9 and *T. jamesi~~s~~i* where *Trebouxia* sp. TR9 performed better under high temperatures, indicating a different capacity to adaptation.

~~Under controlled experimental conditions, *Trebouxia* strains tested in this study showed characteristic profiles of endogenous content in phytohormones (Fig. 6). IAA levels found in *Trebouxia* sp.*'arnoldoi'* (Fig. 6a) are similar to those observed in the lichen forming algae *Trebouxia* sp., *Trebouxia decolorans* and *Asterochloris glomerata* grown in solid media (Pichler et al. 2020). ABA production has been also detected in other isolated lichen forming algae, such as *Trebouxia* sp. TR9 (Hinojosa-Vidal et al. 2018) as well in the previously mentioned *Trebouxia* sp., *T. decolorans* and *A. glomerata* (Pichler et al. 2020). The ABA levels observed in the *Trebouxia* strains tested in this study (Fig. 6b) are in the same range of magnitude as the levels in *Trebouxia* sp. TR9, *Trebouxia* sp. and *T. decolorans* (Hinojosa-Vidal et al. 2018; Pichler et al. 2020). In contrast, the endogenous bioactive form of JA, JA-Ile (Fonseca et al. 2009), is also found in all three *Trebouxia*~~

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~~strains from *B. zoharyi* (Fig. 6d), while no detectable endogenous levels of JA have been found in *Trebouxia sp., T. decolorans and A. glomerata* (Pichler et al. 2020). In addition, SA and Me-JA were also detected in the *Trebouxia* strains isolated in this study. Regarding these phytohormones, there is an absence of previous studies that describe their presence in lichen-forming algae, although Me-JA has been detected in *Chorella* (Ueda et al. 1991).~~

~~The different phytohormone profiles obtained in the three *Trebouxia* strains isolated in this study (Fig. 6f), and in the other lichen forming algae mentioned above, suggest that each microalgae strain could present different internal signalling needs, which could be caused by the adaptation to a different symbiotic environment in each lichen thalli. On the other hand, it cannot be ruled out that a part of the production of these phytohormones could also be secreted to the external environment and play a role in external signalling mechanisms with the other members of the thallus symbiotic system. Related to this, the extracellular release of IAA, ABA and JA in the lichen-forming algae *Trebouxia sp., T. decolorans* and *Asterochloris glomerata*, has recently been described (Pichler et al. 2020). The determination of the role of these phytohormones in the internal signalling mechanisms of the phycobionts, as well as in the coordination mechanisms with the rest of the elements of the lichen symbiosis, is a question that needs future studies.~~

Our results indicate that *B. zoharyi* is flexible regarding the photobiont choice depending on the region, and suggest that bioclimatic factors could influence the myco/photobiont association patterns due to the different photosynthesis response to different temperatures (20º C and 17º C). ~~Our results clearly indicate that association patterns in each Island could be driven by phycobiont physiological features and/or mycobiont specialization (Ortiz-Álvarez et al. 2015).The mycobiont provides a "growth chamber" (Honegger 2009) with suitable conditions for physiological activity of the photosynthetic partner. However, special adaptations in the physiology of the phycobiont are postulated to explain the ability of the phycobiont to cope with severe environmental conditions. The results point to habitat-specific adaptations which lead to similar behaviour in phycobionts which are genetically different. The physiological adaptations of lichen phycobionts may substantially contribute to the stress resistance strategy and the colonization capacity. This may be particularly relevant in changing conditions predicted by the climate-change scenario.~~

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Fig. 1 *Trebouxia* phylogenetic analysis. Rooted ITS1-5.8S-ITS2 + LSU rDNA gene tree representing 41 *Trebouxia* sequences, including 28 well- accepted *Trebouxia* species from SAG and UTEX, *Trebouxia* sp. TR9 and *Trebouxia crespoana* from ASUV retrieved from the GenBank. Newly generated sequences are marked as ex. *Buellia zoharyi*_locality_haplotype code_frequency.

Three *Trebouxia* species detected were indicated. Values at nodes indicate statistical support estimated by two methods: bootstrap support (BS, RAxML analysis) and posterior probabilities (PP, MrBayes analysis). Scale bar shows the estimated number of substitutions per site.

Fig. 2 a, b Cross-section of *B. zoharyi* thalli from Lanzarote by TEM. **a** ~~Phycobiont~~Photobionts of *B. zoharyi* inside thallus, **b** Detail of pyrenoid. **c, d** Cross-section of *B. zoharyi* thallus from Fuerteventura by TEM. **c** ~~Phycobiont~~Photobionts of *B. zoharyi* inside thallus, **d** Detail of pyrenoid. **e, f** Cross-section of *B. zoharyi* thalli from Tenerife by TEM. **e** ~~Phycobiont~~Photobionts of *B. zoharyi* inside thallus, **f** Detail of pyrenoid. Abbreviations: Py, Pyrenoid; Pg, Pyrenoglobuli; Chl, Chloroplast; PV, Peripheral vesicles. Bars 500 nm, 800 nm, 1 µm and 2 µm.

Fig. 3 Algal multiplicity detected by PCR depicted in the localities included in this study: Tenerife, Lanzarote and Fuerteventura. Inner, outer circle and numbers represent the primary and secondary ~~phycobiont~~photobiont detected, respectively. The colour coding for each *Trebouxia* is shown on the bottom left-hand side of the figure.

Fig. 4 Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20º C. Three cultures were sampled for each condition and phytohormone levels were quantified: **a** indole-3-acetic acid (IAA); **b** abscisic acid (ABA); **c** salycilic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and **e** methyl jasmonate (Me-JA). Graphs show the mean ± standard deviation. Significant differences between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis of the phytohormone content of each Trebouxia strain. Scale bar (Z-score) indicates the relative abundance of a particular phytohormone in each strain.

Fig. 5~~4~~ a Maximum fluorescence yield (F_v / F_m) of the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. Bars represent means ± SE, n = 4. Significant differences between treatments and species are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **b** Chlorophyll fluorescence images for Fv/Fm of the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. The color scale bar shown at the bottom of the figures stands for values from 0 (black) to 1 (pink).

Fig. 6~~5~~ a PPFD response curves of the non-photochemical quenching (NPQ), **b** relative electron transport rate (ETR), and **c** PSII photooxidative pressure (1-qP) in the different *Trebouxia* strains isolated from *Buellia zoharyi* growing in BBM medium at 17º C and 20º C. Bars represent means ± SE, n = 4. ~~Two areas of interest (AOI) were selected for Chl fluorescence measurements, one in the central part and one in the outer zones in order to evaluate spatial heterogeneity. The values of~~

chlorophyll fluorescence parameters were means of two AOI (internal and external); no significant differences were obtained between internal and external areas.

Fig. 6 Phytohormone profiles of axenic cultures of the different *Trebouxia* strains isolated from *Buellia zoharyi*. Microalgae were grown in BBM medium for 21 days at 20º C. Three cultures were sampled for each condition and phytohormone levels were quantified: **a** indole-3-acetic acid (IAA); **b** abscisic acid (ABA); **c** salycilic acid (SA); **d** jasmonic acid conjugated to isoleucine (JA-Ile) and **e** methyl jasmonate (Me-JA). Graphs show the mean ± standard deviation. Significant differences between strains are indicated with letters (ANOVA, Tukey HSD test, $p < 0.05$). **f** Heatmap analysis of the phytohormone content of each Trebouxia strain. Scale bar (Z-score) indicates the relative abundance of a particular phytohormone in each strain.