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Trebouxia maresiae sp. nov. (Trebouxiophyceae,
Chlorophyta), a new lichenized species
of microalga found in coastal environments

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***Trebouxia maresiae* sp. nov. (Trebouxiophyceae, Chlorophyta), a new lichenized species of microalga found in coastal environments**

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ABSTRACT

Coccoid microalgae of the genus *Trebouxia* Puymaly are by far the most prevalent among the various species involved in lichen symbioses. However, their taxonomic knowledge is rather scarce compared to that of lichenized fungi. In the present work, a taxonomic study integrating diverse techniques (phylogenetics, light, confocal and transmission electron microscopies) is carried out to describe *Trebouxia maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. This species widely associates with the red-listed lichenized fungus *Seirophora villosa* (Ach.) Frödén but also with species of the genus *Ramalina* Ach., both occurring in coastal environments in the western Mediterranean and the Cape Verdean islands. This microalga is circumscribed to *Trebouxia* clade A and is closely related to *T. de-*

KEY WORDS
Mediterranean,
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MOTS CLÉS
Méditerranéen,
Cap-Vert,
lichen,
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symbiose,
espèce nouvelle.

colorans Ahmadjian. It is characterized by the cell size being up to 15 µm in diam., the crenulate chloroplasts, and the structure of pyrenoids, which in cultured cells fits well with the crenulata-type, with long branched tubules meandering through the pyrenoid matrix, whereas in the lichenized state it acquires a hybrid structure (maresiae-type), characterized by the periphery of the pyrenoid being rather gigantea-type, with thylakoid membranes forming short, branched tubules. With the present work, the taxonomy of the genus *Trebouxia* moves a step forward towards more accurately characterizing species in lichen microalgae which is a prerequisite for future, more complex studies on speciation, co-evolution and selectivity.

RÉSUMÉ

Trebouxia maresiae sp. nov. (*Trebouxiophyceae*, *Chlorophyta*), une espèce nouvelle de microalgue lichénisée présente dans les environnements côtiers.

Les microalgues cocoïdes du genre *Trebouxia* Puymaly sont les plus répandues parmi les différentes espèces associées aux symbioses lichéniques. Cependant, la connaissance taxonomique de ces espèces est plutôt lacunaire par rapport à celle des champignons lichénisés. Dans ce travail, une étude taxonomique intégrant diverses techniques (phylogénétique, microscopies optique, confocale et électronique à transmission) a été réalisée pour décrire *Trebouxia maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. Cette espèce s'associe principalement au champignon lichénisé *Seirophora villosa* (Ach.) Frödén, figurant sur la liste rouge, mais aussi avec des espèces du genre *Ramalina* Ach., tous deux présents dans les environnements côtiers de la Méditerranée occidentale et des îles du Cap-Vert. Cette microalgue est circonscrite au clade A de *Trebouxia*, et démontre une relation étroite avec *T. decolorans* Ahmadjian. Elle se caractérise par la taille de ses cellules (jusqu'à 15 µm de diamètre), ses chloroplastes crénelés et la structure de ses pyrénoïdes qui, dans les cellules cultivées, correspond bien au crenulata-type, avec de longs tubules ramifiés serpentant dans la matrice du pyrénoïde, alors qu'à l'état lichénisé, elle adopte une structure hybride (maresiae-type), caractérisée par la périphérie du pyrénoïde étant plutôt du gigantea-type, avec les membranes des thylacoïdes formant des tubules courts et ramifiés. Cette contribution à la taxonomie du genre *Trebouxia* permet de faire avancer l'évaluation plus précise de la délimitation des espèces chez les microalgues lichéniques, ce qui est une condition préalable à des études futures plus complexes sur la spéciation, la co-évolution et la valeur sélective.

INTRODUCTION

The class *Trebouxiophyceae* Friedl (Chlorophyta) comprises green algal lineages with contrasting habitat preferences (aquatic or aerophytes) and with diverse morphological complexities, which range from coccoid or elliptic unicellular forms to filamentous and blade- or colony-forming species. The coccoid unicellular species in the genus *Trebouxia* Puymaly (*Trebouxiaceae* Friedl) are of the utmost significance for terrestrial ecosystems as they engage in mutualistic associations with a plethora of ascomycetes and basidiomycetes (Fungi, Eukarya) (Muggia *et al.* 2020; Bordenave *et al.* 2022). The phenotypic result (holobiomes) of such cyclical symbioses are lichen thalli (Barreno 2013), which are involved in a number of ecosystem-level processes, including the global cycle of carbon (e.g. Cornelissen *et al.* 2007; Elbert *et al.* 2012; Asplund & Wardle 2017). Lichenization represents a successful lifestyle across evolutionary and ecological scales, with c. 20 000 different lichens known worldwide (Margulies & Barreno 2003; Lücking *et al.* 2017). In fact, lichens occupy c. 8 % of the planet surface, and are able to thrive in inhospitable cold and hot deserts, as well as areas with high saline stress (Nash 2008; Bianchi *et al.* 2019; Sancho *et al.* 2019; Raggio *et al.* 2021). While lichen-forming fungi (mycobionts) are obligate symbionts, microalgae (phyco- or chlorobionts)

have also been found free-living (Tschermark-Woess 1978; Bubrick *et al.* 1984; Yung *et al.* 2014).

The number of known species of *Trebouxia* is to some extent controversial if one looks at the existing literature and online databases. Guiry & Guiry (2021) reported 40 species names and 26 taxonomically accepted species in 2018, whereas Muggia *et al.* (2018) mentioned 28 species in their first review, and later updated that figure to 29 (Muggia *et al.* 2020). Currently, the taxonomy of *Trebouxia* is in need of additional revision. Based on a huge assembly of molecular data and molecular species delimitation analyses, Muggia *et al.* (2020) demonstrated that the number of formally described *Trebouxia* represents only a small portion of the real existing diversity. Their estimates raised the number of candidate species to 109–113, which represents an approximate four-fold increase from the current number of described species.

Trebouxia species have been traditionally described on the basis of phenotypical characters, the most important represented by differences in cell size and shape, chloroplast lobe patterns, number and morphology of pyrenoids, autospore formation, and zoospore structure (Archibald 1975; Friedl 1989; Ettl & Gärtnert 2014). However, the genus has been lately shown to host substantial hidden diversity thanks largely to molecular phylogenetics, but only eight recognized lineages have been formally described as new species in the last two

decades (Beck 2002; Voytsekhovich & Beck 2016; Škaloud *et al.* 2018). This is in stark contrast to the hundreds of new taxonomic novelties and rearrangements done in the lichen fungal taxonomy in approximately the same time period (Lücking *et al.* 2017). Isolating algae from lichen thalli and culturing them, which are necessary steps for cell ultrastructure characterization, are probably the major obstacles for the progress of *Trebouxia* taxonomy (Molins *et al.* 2018).

Recently, Garrido-Benavent *et al.* (2022) studied the genetic diversity of the myco- and phycobionts of the red-listed lichen *Seirophora villosa* (Ach.) Frödén (Teloschistaceae Zahlbr., Ascomycota), which usually forms epiphytic thalli on several shrubs in Mediterranean coastal dune ecosystems. They found that one involved microalga was the undescribed *Trebouxia* sp. 'A46' (Muggia *et al.* 2020). In the present study we adhere to the integrative taxonomic approach proposed in previous works (e.g. Molins *et al.* 2018; Muggia *et al.* 2018; Škaloud *et al.* 2018; Muggia *et al.* 2020; Bordenave *et al.* 2022) by combining molecular phylogenetics with light, confocal and transmission electron microscopies, both on cultured and in symbiotic state, to describe this microalga as a new *Trebouxia* species, thereby updating the taxonomy of this genus.

MATERIAL AND METHODS

TAXON SAMPLING

The list of studied lichen specimens that hosted *Trebouxia* sp. 'A46' was reported in Garrido-Benavent *et al.* (2022). Briefly, the dataset consisted of 71 thalli of *Seirophora villosa* and 27 of *Ramalina lacera* (With.) J.R.Laundon that were collected between 2008–2010 in 14 populations across the western Mediterranean basin, including coastal dune ecosystems in the Iberian and Italian peninsulas, the Balearic Islands and Sardinia. The dataset also included a single *Seirophora* sp. specimen collected in the Cape Verdean Island of Santiago in 2017.

ISOLATION AND CULTURE OF THE LICHEN PHOTOBIONT

A *Seirophora villosa* thallus from Es Trenc (Mallorca, Balearic Islands), a locality where all lichen specimens hosted *Trebouxia* sp. 'A46' (Garrido-Benavent *et al.* 2022), was selected for phycobiont isolation using the method described in Chiva *et al.* (2021). This method consists of an initial thallus washing step that removes any surface-dwelling, non-symbiotic algae; a dissection step of the thallus upper cortex with a sterile razor blade; and a third step of direct inoculation of tiny clumps of the algal layer on a Bold's Basal Medium (BBM; Bold 1949; Bischoff & Bold 1963). The media are maintained at 17 °C under 15 µmol/m²s (PPFD) with a 12/12 hours photoperiod. Afterwards, when the phycobiont colony growth is visible to the naked eye, it is transferred onto liquid BBM and incubated under the same conditions. To obtain single strain algal cultures, algae are streaked on BBM plates and, finally, unicellular colonies are transferred and preserved in liquid and/or solid media.

PCR AMPLIFICATION AND SEQUENCING

Marker amplification by PCR was performed directly from isolated colonies using the fast microalgae identification protocol described by Molins *et al.* (2018). A portion of the plastidial RuBisCO large subunit (*rbcL*) was amplified using primers a-chrbcL-203-5'-MPN and a-ch-rbcL-991-3'-MPN (Nelsen *et al.* 2011). The primer pairs nrSSU-1780/ITS4T (Kroken & Taylor 2001; Piercy-Normore & DePriest 2001) and COXIIIf2/COXIIR (Lindgren *et al.* 2014) were selected for amplifying the two nuclear ribosomal internal transcribed spacers (nrITS1 and nrITS2) together with the 5.8S nrDNA subunit, and the COX2 locus. PCR conditions followed Muggia *et al.* (2020). Products were visualized on 2 % agarose gels, purified using the Gel Band Purification Kit (GE Healthcare Life Science, Buckinghamshire, UK), and sequenced with an ABI 3100 Genetic Analyzer using the ABI Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California). Raw electropherograms were manually checked, trimmed and assembled using SeqmanII v.5.07© (Dnastar Inc.). GenBank accession numbers are in Table 1. Data of the plastid-encoded markers 23SU rDNA and *psbA* of the studied microalga were obtained in Garrido-Benavent *et al.* (2022).

SEQUENCE DATASETS CONSTRUCTION, ALIGNMENT AND PHYLOGENETIC ANALYSES

Trebouxia sp. 'A46' was phylogenetically placed within *Trebouxia* clade 'A' (*arboricola/gigantea* group) in Muggia *et al.* (2020), and more specifically into a subclade including *T. aggregata* (Archibald) Gärtner, *T. arboricola* Puymaly, *T. crenulata* Archibald, *T. decolorans* Ahmadjian and *T. solaris* Voytsekhovich & Beck, among other undescribed species. We therefore compiled our sequence datasets considering only the representatives of that subclade. Because those lacked COX2 data, we only constructed the nrITS and *rbcL* datasets. Alignments were carried out in Geneious® v.9.0.2 using MAFFT v.7.222 (Katoh *et al.* 2002; Katoh & Standley 2013) and the following parameters: the FFT-NS-I ×1000 algorithm, the 200PAM/k = 2 scoring matrix, a gap open penalty of 1.5 and an offset value of 0.123. A manual editing of the resulting nrITS alignment consisted of trimming alignment ends coding for 18S and 26S ribosomal subunits, as well as replacing gaps at the ends of shorter sequences with "N", which represents any base. Partitions (i.e., nrITS spacers, 5.8S subunit, and codon positions in *rbcL*) were adequately annotated. Then, the concatenated two-locus dataset was chosen to infer a maximum likelihood (ML) phylogenetic tree with RAxML-NG (Kozlov *et al.* 2019) using the Swiss Institute of Bioinformatics website. We selected the following optimal partitioned evolutionary model according to a preliminary analysis with PartitionFinder v.1.1.1 (Lanfear *et al.* 2012): SYM+Γ (nrITS1+2), JC (5.8S, *rbcL*-2nd codon position), F81 (*rbcL*-1st cod. pos.) and HKY+I (*rbcL*-3rd cod. pos.). Branch lengths were linked and an optimization of topology, branch lengths and model were allowed during the ML tree search. Bootstrapping consisted of a fixed number of replicates (100). Additionally, a phylogenetic reconstruction

TABLE 1. — Details of the *Trebouxia* Puymaly collections used in this study. Species codes starting with the letter “A” are indicated for cross-reference with the study of Muggia et al. (2020).

<i>Trebouxia</i> species	Code	Voucher / Culture collection	GenBank accession no.	
			nrITS	rbcL
<i>T. aggregata</i> (Archibald) Gärtnér	A13	UTEX-180	—	AJ969643
<i>T. arboricola</i> Puymaly		SAG-219	Z68705	AM158960
<i>T. crenulata</i> Archibald		AV020	KT819989	—
<i>Trebouxia</i> sp.		L1184	KJ754304	KM091733
<i>Trebouxia</i> sp.	A36	L101	AJ969540	AJ969664
<i>T. decolorans</i> Ahmadjian	A33	P319	AJ970889	AM159504
		UTEX-B781	FJ626728	—
<i>Trebouxia</i> sp.	A40	P10	AJ969515	AJ969659
<i>T. maresiae</i> sp. nov.	A46	TSV1	FJ792797	—
		P280	AJ969583	AJ969660
<i>Trebouxia</i> sp.	A48	ASUV 142 (Type)	MZ724411	MZ687825
		P287	AJ969586	AJ969649
		P288	AJ969587	AJ969650
		P303	AJ969589	AJ969667
		P133	AJ969551	AM158961
		P164	AJ969562	—
		P104	AJ969541	AJ969651
<i>T. solaris</i> Voytsekhovich & A. Beck	A35	AV060	KT819918	—
		L1383	KJ754240	KM091793
		P121	AJ969550	AM158967
<i>Trebouxia</i> sp.	A37	L1107	KJ754236	—
<i>Trebouxia</i> sp.	A50	L1382	KJ754239	—
<i>Trebouxia</i> sp.	A51	P400	AM159210	—
<i>Trebouxia</i> sp.	A24	4088	KR913257	—

using the same dataset and partitioned evolutionary model was conducted with the program MrBayes v.3.2.7 that operates under a Bayesian inference context (Ronquist et al. 2012). The analysis considered two parallel, simultaneous four-chain runs executed over 5×10^7 generations starting with a random tree, and sampling after every 500th step; the first 25 % of data was discarded as burn-in, and the 50 % majority-rule consensus tree, and corresponding posterior probabilities (PP), were calculated from the remaining trees. Chain convergence was assessed by ensuring that values of the average standard deviation of split frequencies (ASDSF) dropped below 0.005, and values of the potential scale reduction factor (PSRF) approached 1.00. Tree nodes with bootstrap support (BS) values equal or higher than 70 % and Bayesian posterior probabilities (PP) equal or higher than 0.95 were regarded as significantly supported. Adobe Illustrator CC 2021 was used to manually edit the tree.

LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

A Nikon Eclipse E-800 microscope equipped with a Nikon DS-Ri1 camera was employed to observe and take microphotographs of the microalgae of the Balearic *Seirophora villosa* specimen using differential interference contrast (LM-DIC). Transmission electron microscopy (TEM) examinations were performed on a portion of the thallus mentioned above, as well as on microalgae growing on axenic culture. Algal cells were fixed in 2 % Karnovsky fixative for 12 hours at 4 °C and washed three times for 15 minutes with 0.01 M PBS (pH 7.4), then post-fixed with 2 % OsO₄ in 0.01 M PBS (pH 7.4) for 2 hours at room temperature. Afterwards, they were washed three times in 0.01 M PBS (pH 7.4) for 15 min-

utes and then dehydrated at room temperature in a graded series of ethanol solutions, starting at 50 % and increasing to 70 %, 95 % and 100 % for no less than 20-30 minutes at each step. The fixed and dehydrated samples were embedded in Spurr’s resin according to the manufacturer’s instructions (<http://www.emsdiasum.com/microscopy/technical/datasheet/14300.aspx>). Sections of 90 nm were cut with a diamond knife (DIATOME Ultra 45°) using a Reichert Ultracut E ultramicrotome, later mounted on oval hole copper grids coated with formvar and post-stained with 2 % (w/v) aqueous uranyl acetate and 2 % lead citrate, using the “SynapTek Grid Staining Kit” (<http://www.emsdiasum.com/microscopy/technical/datasheet/71175.aspx>). Finally, sections of the microalgae in cultured and symbiotic states were observed with a Hitachi HT7800 (120 kV) electron microscope fitted with a high-speed CMOS camera (EMSI XAROSA). DIC and TEM examinations were carried out at ICBiBE and SCSIE microscopy services of the University of Valencia.

LASER SCANNING CONFOCAL MICROSCOPY (LSCM)

The 3-dimensional chloroplast morphology was observed by chlorophyll autofluorescence using an Olympus FLUOVIEW FV1000 confocal laser scanning microscope under a 405 nm excitation laser. Fluorescence emitted from 650 to 750 nm was collected to observe chlorophyll autofluorescence. A series of images were captured with a separation of 0.4 µm. The image stack was preprocessed to remove noise and then analyzed with the Fiji distribution of ImageJ, using the z-projection and the volume viewer tools (Schindelin et al. 2012; Rueden et al. 2017).

RESULTS

ALIGNMENT STATISTICS AND PHYLOGENETIC ANALYSES

The nrITS, 5.8S and *rbcL* datasets summed up to 23, 23 and 13 sequences, and had a length of 557, 155 and 1150 bp, respectively. The number of variable sites (informative sites in parentheses) in the corresponding alignments were 62 (38), 2 (1), and 16 (15). The ML analysis in RAxML-NG estimated a topology with a final LogLikelihood of -4105.9893. The MrBayes analysis reached an ASDSF value of 0.005 after 1.92×10^6 generations, and Estimated Sample Sizes (EESs) were well above 200 for all parameters. Because the ML and MrBayes topologies showed no supported conflicts, the topology inferred with MrBayes is presented in Figure 1. It revealed *Trebouxia* sp. 'A46' close to *T. decolorans* and another undescribed species (*Trebouxia* sp. 'A40'), although the group containing all three *Trebouxia* lacked nodal support. *Trebouxia* sp. 'A48' was closely related to all previous three species but again without nodal support. These taxa together with *T. solaris* and four additional undescribed taxa formed a well-supported clade (PP = 1; BS = 100 %). At the nucleotide level, the three specimens of *Trebouxia* sp. 'A46' differed just by the length of a repeated thymine in the nrITS1 region, two times in the specimen TSV1 (FJ792797), and three in the other two specimens (ASUV 142, P280).

MORPHOLOGY

Isolated microalgae were morphologically characterized by LM-DIC, TEM and LSCM after 21 days of growth on solid BBM. Algal cells were usually spherical, and their diameter ranged from 6 µm, in young cells, to 11 µm when mature (n = 40; Fig. 2B-D, H). Mature cells showed a single central chloroplast, which occupied most of the cell volume (Fig. 2C, I-J), and displayed a crenulate morphology with branched and elongated, 'tree-like' lobes projected towards the cell wall (Fig. 2I-K). One central pyrenoid of the crenulata-type *sensu* Bordenave *et al.* (2022) was visible in mature cells (Fig. 2B-C, H); this type of pyrenoid is characterized by showing long branched tubules meandering through the pyrenoid matrix with a matrix thicker than the tubules (Fig. 3); occasionally, secondary pyrenoids accompanied the central one (Fig. 2B, K). Pyrenoglobuli were always present in moderate number inside the pyrenoid matrix next to the tubules (Fig. 3). Some cells containing two chloroplasts were also observed (Fig. 2E). Chloroplasts in old cells show an irregular and more unorganized structure with unstacked thylakoid lamellae around the pyrenoid (Fig. 3B, C), sometimes showing starch granules (Fig. 3C). The cytoplasm of old cells contained carotenoids (Fig. 2D). Furthermore, microalgal cells showed a nucleus located laterally between the chloroplast lobes (Fig. 3D). The bilayer cell wall was 0.21-0.43 µm wide (Fig. 3A-E). Asexual reproduction by means of autospores (4-32) was observed (Fig. 2F-H, L); autosporangia were spherical and sometimes deformed by the growing autospores. Zoospores were not observed. Sexual reproduction was not observed.

When lichenized, cells of this microalga varied morphologically compared to those grown in culture. Thus, their shape

was mostly oval and in general between 8-9 µm in diameter (Fig. 3D-E). The periphery of the pyrenoid also showed a slight deviation from the common crenulata-type. Therefore, this hybrid pyrenoid, described here as maresiae-type, is characterized by showing two distinct regions: a central region with long branched tubules meandering through the pyrenoid matrix, with a matrix thicker than the tubules, and with pyrenoglobules present inside the pyrenoid matrix next to the tubules, resembling the crenulata-type pyrenoid; and a peripheral region characterized by short-branched tubules perforating the pyrenoid matrix, with tubules as thick as the matrix, and with pyrenoglobules at the margins of the pyrenoid, resembling the gigantea-type pyrenoid (arrowheads in Fig. 3D-F). Old mature cells were larger in size (13-15 µm), rather spherical, their cell wall thicker (0.65-0.85 µm). A summary of the similarities and differences in cell and chloroplast characters between the new species and closely related ones is shown in Table 2. According to Honegger (1986), the type of fungal-algal interaction corresponded mostly to wall-to-wall appositions through the formation of appressoria (Fig. 3E).

Family TREBOUXIACEAE Friedl
Genus *Trebouxia* Puymaly

Trebouxia maresiae
Garrido-Benavent, Chiva & Barreno, sp. nov.
(Figs 1-3)

Trebouxia maresiae Garrido-Benavent, Chiva & Barreno, sp. nov. shows crenulate chloroplasts that bear a crenulata-type pyrenoid when cells are in culture, whereas in the lichenized state it acquires a hybrid structure (maresiae-type), characterized by the periphery of the pyrenoid being rather gigantea-type, with thylakoid membranes forming short, branched tubules. These traits distinguish the new species from *T. decolorans*, which has deeply lobed chloroplasts with a decolorans-type pyrenoid. The two species also differ by the relatively high number of nucleotide differences in their nrITS DNA sequences. Vegetative cells of *T. maresiae* sp. nov. tend to be smaller in diameter (< 15 µm) than those of other species in *Trebouxia* clade A like *T. decolorans* and *T. solaris*.

HOLOTYPE. — Spain. Balearic Islands, Mallorca, Es Trenc, phytophagous of *Seirophora villosa* (Ach.) Frödén collected on branches of *Juniperus phoenicea* subsp. *turbinata* (Guss.) Nyman in coastal dunes, 39°20'40.71"N, 2°59'8.24"E, 3 m a.s.l., 07.VIII.2008, leg. G. Salvà. The lichen specimen was deposited in the herbarium of the University of Valencia (VAL-Lich) with the following code: VAL-Lich 31798.

TYPE STRAIN. — Deposited at the Symbiotic Algal collection from the University of Valencia (ASUV), as item type ASUV 142.

REFERENCE STRAINS. — ASUV 142 and ACOI 3420, deposited at the Coimbra Collection of Algae (ACOI), University of Coimbra.

GENBANK ACCESSION NUMBERS. — MZ724411 (nrITS), MZ687825 (*rbcL*) and MZ687826 (COX2).

ETYMOLOGY. — According to the Canarian Academy of Language, the term "maresía", from which the specific epithet "maresiae" derives, means "air laden with marine humidity in areas close to the

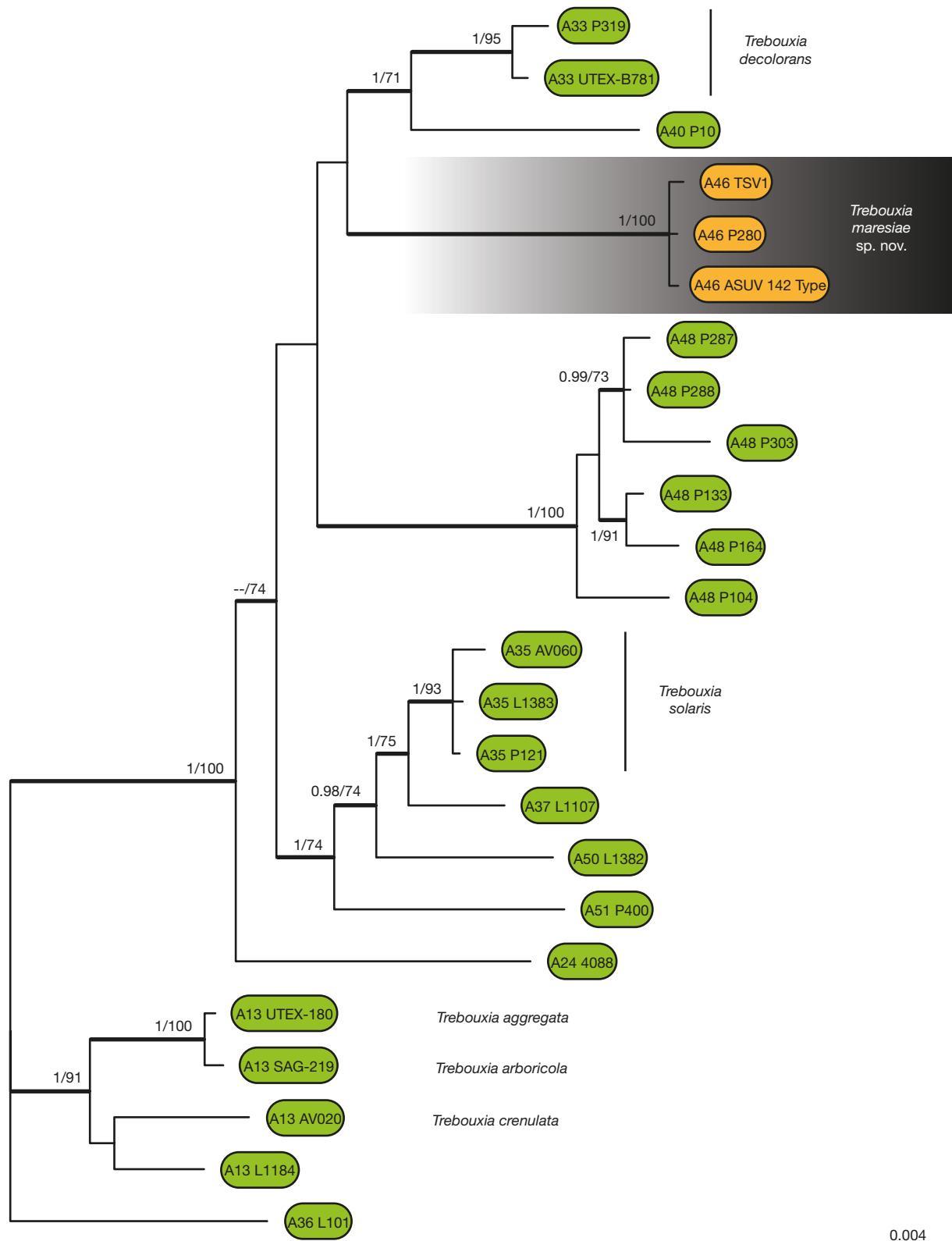


FIG. 1. — Phylogram showing the placement of the new species in a subclade of *Trebouxia* Puymaly clade A sensu Muggia et al. (2020). The phylogeny was built with MrBayes based on a three-locus dataset (nrITS, 5.8S and *rbcL*). For reference, green- and orange-filled boxes on the right of each tip indicate the species code of each *Trebouxia* species following Muggia et al. (2020). To the right, the voucher or culture collection code, as well as the name of the species, if any, are provided. Posterior Probabilities (PP) and bootstrap support values (BS, RAxML-NG analysis) are represented on branches leading to nodes on the left and right, respectively. Branches in **bold** had a significant statistical support (PP ≥ 0.95; BS ≥ 70 %).

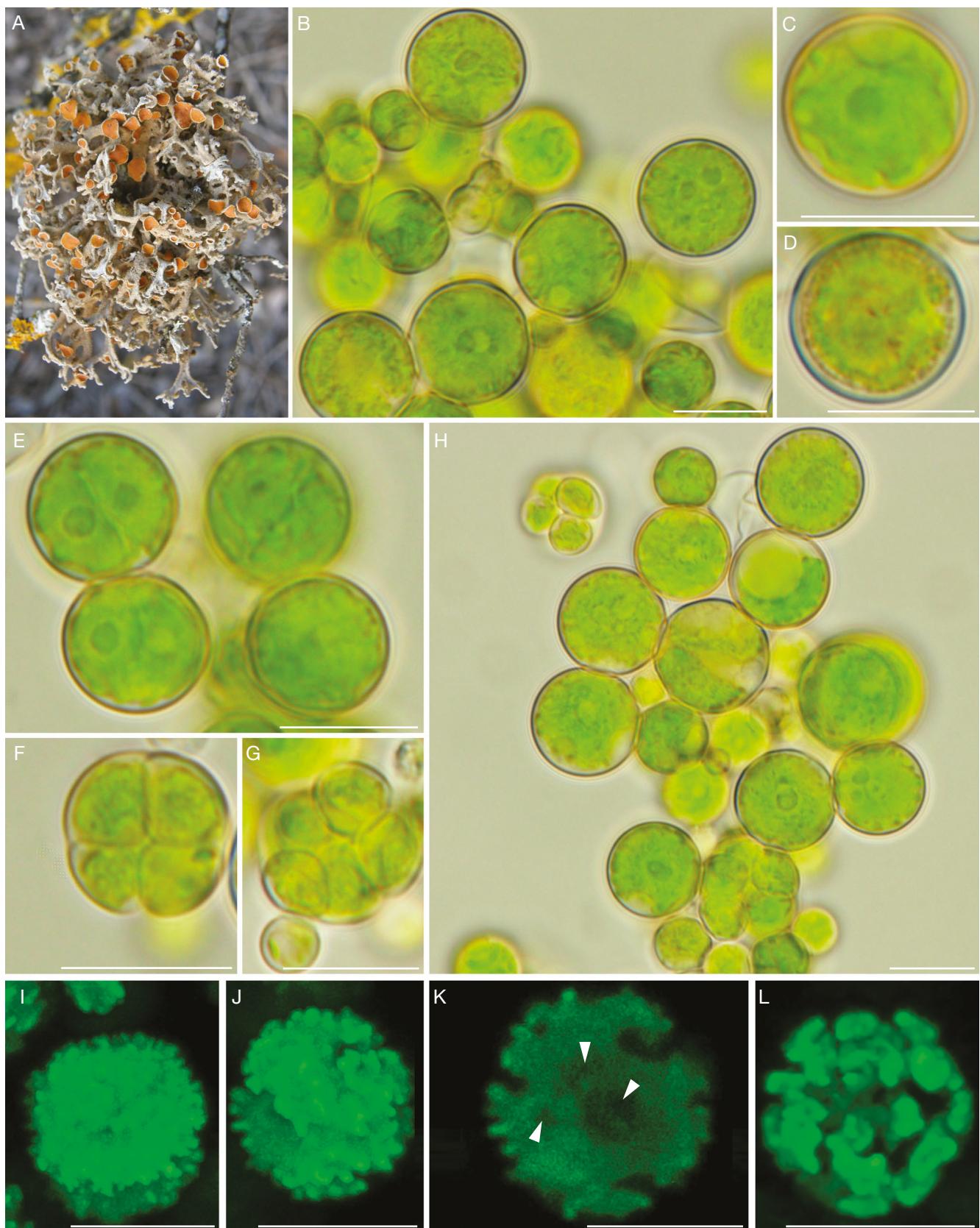


FIG. 2. — Habitat and morphology of *Trebouxia maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov.: A, a thallus of the lichen *Seirophora villosa* (Ach.) Frödén from Es Trenc (Mallorca) with which this microalga associates; orange discs are apothecia produced by the lichen fungus to reproduce sexually; B–H, light microscopy photographs showing the gross morphology of vegetative cells and autosporangia; B, H, young and mature cells; C, central, crenulate chloroplast with a single pyrenoid; D, old cell with carotenoids in the cytoplasm; E–G, development of autosporangia and autospores; I–K, reconstruction of chloroplasts by LSCM; K, crenulate chloroplast with three pyrenoids (white arrowheads); L, reconstructed chloroplast of autospores within an autosporangium. Scale bars: 10 µm.

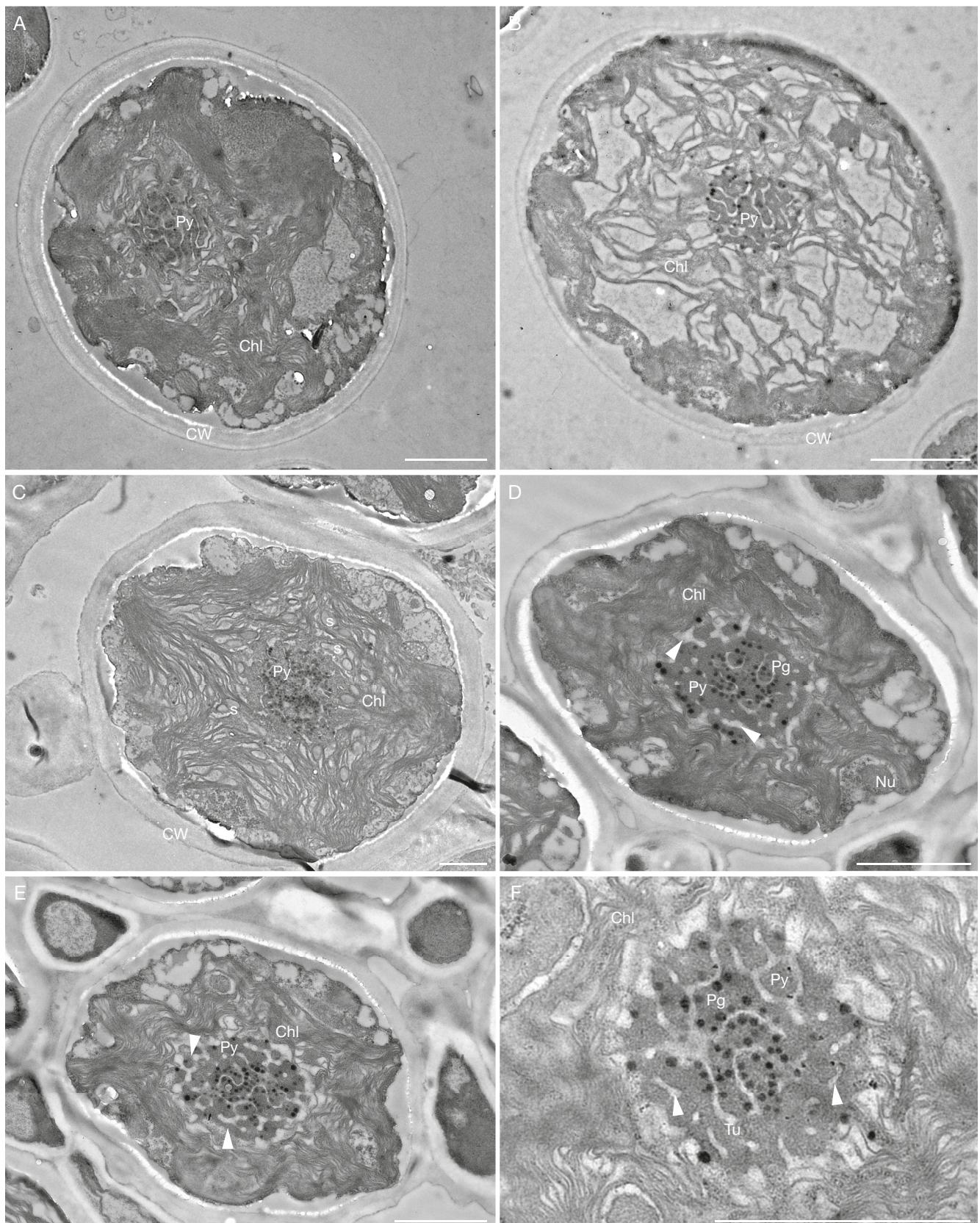


Fig. 3. — Ultrastructure of *Trebouxia maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. by TEM: A, B, cultured cells; C-F, cells within lichen thallus; F, detail of a pyrenoid. **White arrowheads** in D, E and F indicate the tubules in pyrenoid periphery that are more similar to these which characterize the gigantea-type pyrenoid. Abbreviations: Chl, chloroplast; CW, cell wall; Pg, pyrenoglobuli; Py, pyrenoid; Tu, tubules; Nu, nucleus; s, starch granules. Scale bars: 2 µm.

TABLE 2. — Similarities and differences in vegetative cell and chloroplast morphologies, and pyrenoid ultrastructure between the new species of *Trebouxia* Puymaly (**in bold**) and closely related ones.

<i>Trebouxia</i> species	<i>T. aggregata</i>	<i>T. arboricola</i>	<i>T. crenulata</i>	<i>T. decolorans</i>	<i>T. maresiae</i> sp. nov.	<i>T. solaris</i>
Shape and size (diameter) of mature, vegetative cells	Spherical, 8-16 µm	Spherical, 13-15 µm	Spherical, 5-18 µm	Spherical, 19-25.5 (30) µm	Spherical, 7-11 (15) µm	Spherical, 15-20 (22) µm
Chloroplast morphology	Crenulate, with slightly branched lobes	Crenulate, with slightly branched lobes	Crenulate, with tree-like branched lobes	Deeply lobed	Crenulate, with tree-like branched lobes	Crenulate
Pyrenoid number and type	Single, gigantea-type pyrenoid	Single, gigantea-type	Single, crenulata-type	Multiple, decolorans-type	Single, maresiae-type	Single pyrenoid, type not assessed

seashore". Therefore, this term refers to the particular habitat where the lichen phycobiont lives, in coastal sea-shore environments.

ECOLOGY. — In association with lichen-forming fungi of the genera *Seirophora* Poelt, *Ramalina* Ach. (this work), and *Xanthoria parietina* (L.) Th. Fr. (isolate no. P-280-II-a-Sc; Nyati *et al.* 2014).

DISTRIBUTION. — Currently, the known geographic distribution of the new alga includes the Mediterranean basin and the Cape Verdean Island of Santiago (this study; Nyati *et al.* 2014; Garrido-Benavent *et al.* 2022).

DESCRIPTION

Cells are mostly unicellular, rarely in tetrads and octads. Vegetative mature cells usually spherical, rarely oval, 7-11 (15) µm diam. Cell wall is usually thin and arranged in a bilayer. Chloroplast central, dissected into crenulated lobes with several tree-like lobes elongated at their ends. Some cells containing two chloroplasts may be also observed. The chloroplast usually contains a single pyrenoid of the crenulata-type with wide, branched tubules of curved profile, often crossing the entire pyrenoid. In symbiosis, however, the pyrenoid shows a hybrid structure (maresiae-type), characterized by displaying two discrete regions that can be each clearly assigned to different pyrenoid types; here, the pyrenoid periphery is more similar to the gigantea-type. Secondary pyrenoids may be present. Pyrenoglobuli present in the closest parts of the chloroplast stroma. The cytoplasm of old cells is sometimes yellowish, probably due to the presence carotenoid pigments, and vacuolated. Asexual reproduction involves the formation of autospores. Autosporangia (10-11 µm diam.) usually contain 4-16 (32) autospores. Sexual reproduction was not observed.

DISCUSSION

The described species *Trebouxia maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. has been found in association with the fruticose-forming lichen genera *Seirophora* (Teloschistaceae) and *Ramalina* (Ramalinaceae) across the western Mediterranean area, i.e., the Iberian and Italian peninsulas, Sardinia and the Balearic Islands. Previously, Nyati *et al.* (2014) revealed this alga in symbiosis with the very common teloschistaceous, foliose lichen *Xanthoria parietina* (L.) Beltr. in Mallorca (Balearic Islands). The phycobiont of an epiphytic *Seirophora* sp. from

Cape Verde found at c. 900 m of altitude is also included in the concept of *T. maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov., although it showed a slight genetic differentiation (Garrido-Benavent *et al.* 2022). Taken together, these data allow two predictions to be made that will, however, require additional research: 1) the global distribution of this micro-alga extends out of the Mediterranean Basin and over the Macaronesian Region; and 2) the microalga is well adapted to environments with a persistent marine influence that are characterized by displaying high salt concentration and rapid desiccation-rehydration cycles (Le Devehat *et al.* 2014).

The proximity of *T. maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. to *T. decolorans* may be summarized in three items. First, our two-loci phylogenetic reconstruction revealed a close but not statistically supported relationship between them, and showed their membership to *Trebouxia* clade A (Beck 2002; Helms 2003), which currently includes most undescribed *Trebouxia* species (Muggia *et al.* 2020). Overall, the species shows a pattern of intraspecific variation across its geographic distribution (Garrido-Benavent *et al.* 2022) in a similar way to the genetic variation observed in *T. decolorans* (Werth & Sork 2010; Dal Grande *et al.* 2014; Catalá *et al.* 2016). Second, both species use to associate with lichen fungi of the phylogenetically unrelated families Teloschistaceae and Ramalinaceae C. Agardh. In fact, *T. decolorans* is a common phycobiont of *Ramalina menziesii* Taylor and *R. fraxinea* (L.) Ach. (Werth & Sork 2010; Catalá *et al.* 2016). Finally, cells of the two species are usually spherical or globose (Nyati *et al.* 2014). However, *T. decolorans* displays a decolorans-type pyrenoid and deeply lobed chloroplasts (Bordenave *et al.* 2022), whereas *T. maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. shows crenulate chloroplasts that bear a crenulata-type (in culture) or maresiae-type (in symbiosis) pyrenoid. A formal description of the hybrid pyrenoid of the new species has been made in the results section of this paper. Furthermore, vegetative cells of the new species tend to be smaller in diameter when mature compared to *T. decolorans* as well as to other related species, such as *T. solaris*.

In conclusion, *T. maresiae* Garrido-Benavent, Chiva & Barreno, sp. nov. stands out as a study model for intraspecies modification of the pyrenoid as well as for its ability to thrive in high salt concentration environments such as coastal dune systems.

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