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Molecular phylogeny and ultrastructure of the lichen microalga *Asterochloris mediterranea* sp. nov. from Mediterranean and Canary Islands ecosystems

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The microalgae of the genus *Asterochloris* are the preferential phycobionts in *Cladonia*, *Lepraria* and *Stereocaulon* lichens. Recent studies have highlighted the hidden diversity of the genus, even though phycobionts hosting species of the genus *Cladonia* in Mediterranean and Canarian ecosystems have been poorly explored. Phylogenetic analyses were made by concatenation of the sequences obtained with a plastid – LSU rDNA – and two nuclear – internal transcribed spacer (ITS) rDNA and actin – molecular markers of the phycobionts living in several populations of the *Cladonia convoluta-Cladonia foliacea* complex, *Cladonia rangiformis* and *Cladonia cervicornis* s. str. widely distributed in these areas in a great variety of substrata and habitats. A new strongly supported clade was obtained in relation to the previously published *Asterochloris* phylogenies. Minimum genetic variation was detected between our haplotypes and other sequences available in the GenBank database. The correct identification of the fungal partners was corroborated by the ITS rDNA barcode. In this study we provide a detailed characterization comprising chloroplast morphology, and ultrastructural and phylogenetic analyses of a novel phycobiont species, here described as *Asterochloris mediterranea* sp. nov. Barreno, Chiva, Moya et Škaloud. A cryopreserved holotype specimen has been deposited in the Culture Collection of Algae of Charles University in Prague, Czech Republic (CAUP) as CAUP H 1015. We suggest the use of a combination of several nuclear and plastid molecular markers, as well as ultrastructural (transmission electron and confocal microscopy) techniques, both in culture and in the symbiotic state, to improve novel species delimitation of phycobionts in lichens.

Abbreviations: BI, Bayesian inference; CBC, compensatory base change; CM, confocal microscopy; ITS, internal transcribed spacer; LM, light microscopy; ML, maximum-likelihood; SEM, scanning electron microscopy; TEM, transmission electron microscopy; wMP, weighted maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the ITS1-5.8S rRNA gene-ITS2-26S rRNA gene region of *Asterochloris mediterranea* are KP257366 to KP257398, those for the LSU rDNA gene are KP257300 to KP257332, and those for the actin gene are KP257333 to KP257365. The GenBank/EMBL/DDBJ accession numbers for the ITS1-5.8S rRNA gene-ITS2 region of the *Cladonia convoluta-Cladonia foliacea* complex, *Cladonia rangiformis and Cladonia cervicornis* s. str. are KP257399 to KP257424.

Two supplementary tables and one supplementary figure are available with the online Supplementary Material.

INTRODUCTION

Lichens exemplify the details of complex individuality since they are the outcome of cyclical obligate associations involving at least two very different organisms, a heterotrophic fungus (mycobiont) and a photoautotrophic (photobiont) cyanobacterium (cyanobiont) or/and a unicellular green alga (phycobiont, chlorobiont) (Barreno, 2013). Lichenization allows the partners to thrive in habitats that would otherwise be unavailable to either one on its own, and they are frequently successful in outperforming vascular plants, and even bryophytes, in terms of biodiversity as well as biomass. Lichens also host diverse and heretofore little explored communities of non-phototrophic lichenic bacteria

(Aschenbrenner *et al.*, 2014). Several patterns for mycobiont–phycobiont interactions have been described, but the underlying mechanisms may differ considerably depending on the lichen species. Most of the studies on population structure have reported the presence of a single primary phycobiont species per thallus (Yahr *et al.*, 2004; Muggia *et al.*, 2008; Nelsen & Gargas, 2008) or multiple phycobiont genotypes in a single thallus (Ohmura *et al.*, 2006; Dal Grande *et al.*, 2014; Muggia *et al.*, 2014; Nyati *et al.*, 2014). Additional complexity has been reported (Casano *et al.*, 2011; del Campo *et al.*, 2013; Molins *et al.*, 2013) inside a single lichen thallus by the intrathalline coexistence of different algal species and/or genera.

Because of the obligate and intimate relationship between the photobionts and the mycobiont, it has been hypothesized that lichen symbioses undergoes long-term coevolution or concerted diversification (Ahmadjian, 1987; Rambold *et al.*, 1998; del Campo *et al.*, 2013). On the other hand, no overall co-speciation was evidenced between algal and fungal partners in the worldwide-distributed genus *Cladonia* (Piercey-Normore & DePriest, 2001). This genus represents one of the largest genera of lichen-forming fungi with more than 400 described species (Ahti, 2000). Species of the genus *Cladonia* are often major contributors to overall biomass in diverse habitats and ecosystems (Lechowicz & Adams, 1974; Munger *et al.*, 2008). Moreover, *Cladonia* thalli are among the most complex of lichens, and the interpretation of phenotypic variation of their thalli has been controversial (Stenroos & DePriest, 1998; Stenroos *et al.*, 2002a, b; Grube & Hawksworth, 2007). Several recent molecular studies have revealed a lack of correlation between morphological and molecular data, and many traditionally well-delimited species seem to be problematic (Myllys *et al.*, 2003; Kotelko & Piercey-Normore, 2010; Piercey-Normore *et al.*, 2010; Pino-Bodas *et al.*, 2012a, b, c; Steinová *et al.*, 2013).

Cladonia represents a genus known for its prevailing specificity to *Asterochloris* algae (Piercey-Normore & DePriest, 2001; Nelsen & Gargas, 2006, 2008). This genus of microalga has been studied recently from the lichen fungi in the Cladoniineae (genera *Cladonia*, *Lepraria*, *Stereocaulon*) and in *Diploschistes muscorum*, a common parasite of species of the genus *Cladonia* (Škaloud & Peksa, 2010; Škaloud *et al.*, 2015). Phylogenetic analysis based on the concatenated set of internal transcribed spacer (ITS) rDNA and actin type I intron sequences revealed 20 well-resolved clades among *Asterochloris* phycobionts and particular clades were found to be associated with taxonomically different, but ecologically similar, lichens. Additional large hidden diversity in *Asterochloris* lineages was revealed in species of the genus *Cladonia* from India (Řídká *et al.*, 2014).

Piercey-Normore & DePriest (2001) showed that there are very few algal genotypes shared among variously related taxa of the family Cladoniaceae, implying that selectivity is not equal between lichen-forming fungi and algae. Contrasting findings suggested that different patterns of selectivity and specificity may occur in different lichen taxa (Piercey-Normore, 2004; Yahr *et al.* 2004; Nelsen & Gargas, 2008, 2009; Fernández-Mendoza *et al.*, 2011; Magain & Sérusiaux, 2014). Subsequent analyses (Peksa & Škaloud, 2011) revealed that these *Asterochloris* phycobionts could exhibit clear preferences for environmental factors. These algal preferences may limit the ecological niches available to lichens and lead to the existence of specific lichen guilds. Likewise, recent physiological studies evidenced that dehydration rate and time of desiccation affect recovery in *Asterochloris erici* cultures; in addition proteomic and transcript analyses suggest that desiccation tolerance seems to be achieved by constitutive mechanisms in this alga (Gasulla *et al.*, 2009, 2013).

Similar to other characteristics, the secondary structure of rRNA has also been used in evolutionary comparisons (Coleman *et al.*, 1998; Lott *et al.*, 1998; Hausner & Wang, 2005). Patterns in the secondary structure of the ITS rRNA transcripts in *Asterochloris* and in other microalgae have been used as an additional attribute to delimit species boundaries (Beiggi & Piercey-Normore, 2007; Škaloud & Peksa, 2010; Škaloudová & Škaloud, 2013). However, it was demonstrated that differences in the ITS rRNA secondary structures are often not diagnostic at the species level in green algae (Caisová *et al.*, 2011; Škaloud & Rindi, 2013).

This work was focused on four species of the genus *Cladonia*: *Cladonia foliacea* (Huds.) Willd., which is the most common and widely distributed species in the Mediterranean area, sharing its habitat with *Cladonia convoluta* (Lam.) Anders; *Cladonia rangiformis* Hoffm. and *Cladonia cervicornis* s. str. (Ach.) Flot. (Pino-Bodas *et al.*, 2013a). All of these taxa grow preferentially in scrublands and forest clearings under prevailing dry bioclimates (Burgaz & Ahti, 1992, 2009). Several studies (Burgaz & Ahti, 1992, 2009; Litterski & Ahti, 2004) have reported the controversy concerning how to morphologically, chemically and phylogenetically identify the differences between *C. convoluta* and *C. foliacea*; up to the present, it has been impossible to separate them into monophyletic groups, and the term *C. convoluta-C. foliacea* complex has been established (Pino-Bodas *et al.*, 2010). The majority of molecular studies of species of the genus *Cladonia* have focused on mycobiont analyses, but the phycobiont has been mostly ignored and is poorly known.

In this study, we describe a novel phycobiont species, *Asterochloris mediterranea* sp. nov., discovered during our investigations into the phycobionts of *Cladonia* and other terricolous genera in the Mediterranean and Canary Islands ecosystems. Molecular and ultrastructural approaches led us to identify, and characterize in detail, this novel species both from thalli and isolated monoclonal cultures. Moreover, a key to the species of the genus *Asterochloris* has been drawn up.

METHODS

Taxa sampling. Specimens of *C. foliacea* (Huds.) Willd. and *C. convoluta* (Lam.) Anders (*C. convoluta-C. foliacea* complex), *C. rangiformis* Hoffm.

and *C. cervicornis* s. str. (Ach.) Flot. were collected from different locations in the Iberian Peninsula and the Canary Islands (Fig. 1; Table S1 available in the online Supplementary Material). Samples were dried out in the shaded open air for 1 day and then stored at −20 °C until needed.

Isolation and cultivation of phycobionts. The algal symbionts were isolated by the thallus fragmentation method (Ahmadjian, 1993; Peksa & Škaloud, 2008) as follows: small fragments of lichen thalli were plated onto 2 % agar slants of Bold's Basal Medium (BBM) as modified by Bischoff & Bold (1963). The Petri dishes with thalli fragments were incubated at 18 °C, under an illumination of 20–30 mmol m^{-2} s^{-1} and a 16 : 8 h light/dark cycle. If fungal contamination occurred during the cultivation, the contaminants were carefully removed or the thalli fragments were transferred to new plates. After 2–3 weeks, groups of dividing algal cells were observed associated with some of the fragments. To obtain unialgal cultures, small populations of phycobionts were transferred onto the fresh BBM agar slants and incubated accordingly.

Microscopic investigations. Light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques were performed for morphological analysis of 'in thallus' lichen phycobionts. Pieces of rehydrated *C. convoluta-C. foliacea* complex thalli from Fuentidueña de Tajo (Madrid) C6 and Bujaraloz (Zaragoza) C16 were used to examine the *Asterochloris*-type phycobionts inside the thalli. To analyse the morphology of chloroplasts in isolated phycobionts, confocal microscopy (CM) was used. TEM and SEM examinations were made at the SCSIE Service of the University of Valencia.

Light microscopy (LM). For LM analyses, 15–20 μM sections were cut from frozen samples with a sliding microtome (Leica SM 2000R). The sections were observed with an Olympus BX40 microscope equipped with an Olympus SC100-10.6 camera.

Transmission electron microscopy (TEM). For TEM, the cells were fixed in 2 % Karnovsky fixative for 6 h at 4 °C, and washed three times for 15 min with 0.01 M PBS, pH 7.4, and post-fixed with 2 % OsO_4 in 0.01 M PBS, pH 7.4, for 2 h at room temperature. Thereafter, they were washed three times in 0.01 M PBS, pH 7.4, for 15 min and then dehydrated at room temperature in a graded series of ethanol, starting at 50 % and increasing to 70 %, 95 % and 100 % for no less than 20–30 min for each step (Casano *et al.*, 2011; Molins *et al.*, 2013). The fixed and dehydrated samples were embedded in Spurr's resin according to the manufacturer's instructions (Spurr, 1969). Sections (90 nm) were cut with a diamond knife (DiATOME Ultra 45°) using an ultramicrotome (LKB Bromma Nova Ultratome), mounted on copper grids of 100 mesh and post-stained using a SynapTek Grid Staining kit (Electron Microscopy Sciences; http://www.ems-diasum.com/microscopy/technical/datasheet/71175.aspx). The sections were observed with a JEOL JEM-1010 (80 kV) electron microscope, equipped with a MegaView III digital camera and AnalySIS image acquisition software.

Scanning electron microscopy (SEM). SEM was undertaken in order to observe the surface ultrasculpture of the squamules. Fractured

Fig. 1. Distribution of the *Cladonia* samples collected for this study in the Iberian Peninsula and the Canary Islands. Sites 1 to 13 and the various types of substrate are indicated by symbols: quartzites, siliceous (white square □), limestones, calcareous (black square ■), sandstones, siliceous (black triangle ▶), Miocene gypsum (black circle ●) and volcanic (white discontinuous circle ◌). Details of the sampling sites are given in Table S1.

thalli were attached to the holder, coated with palladium/gold and viewed with a Hitachi S-4800 field emission scanning electron microscope.

Confocal microscopy (CM). A Leica TCS SP2 laser scanning confocal microscope equipped with an argon-krypton laser was used. We applied a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. A series of optical sections through chloroplasts was captured and used for three-dimentional reconstruction of their morphology. The chloroplast reconstructions were produced by the ImageJ 1.34p program (Abràmoff *et al.*, 2004), using the 'Volume viewer' plugin.

DNA isolation, amplification and sequencing. Individuals from localities 1 to 13 (Fig. 1) were analysed after washing performed following the protocol of Muggia *et al.* (2013). Samples were given an alphanumeric code from C1 to C33 as shown in Table 1. Total genomic DNA was isolated and purified using a Dneasy Plant Minikit (Qiagen) following the manufacturer's instructions.

Three algal loci were amplified from genomic DNA from each lichen thallus. As chloroplast genome marker, we studied a region of the LSU rRNA gene by using the algal-specific primers 23SU1 and 23SU2 (del Campo *et al.*, 2010a). We also amplified two nuclear loci encoding the nrITS RNA, using the algal-specific primer nr-SSU-1780 (Piercey-Normore & DePriest, 2001) and the universal primer ITS4 (White *et al.*, 1990), and actin type 1, using the algal-specific primer pair ActinF2 Astero (Škaloud & Peksa, 2010) and a-nu-act1-0818-3′ (Nelsen & Gargas, 2006). The primers used to amplify the nuclear ITS rRNA from the mycobiont were ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990).

Table 1. List of taxa included in this study, code (C1 to C33) used in phylogenetic analysis, and the GenBank accession numbers for the newly determined ITS rDNA, LSU rDNA and actin sequences, locality and type of substrate (see also Fig. 1 and Table S1)

Taxon	Code	GenBank accession number				Locality & type of substrate
		Phycobiont			Mycobiont	
		ITS rDNA	LSU rDNA	Actin	ITS rDNA	
C. conv-fol complex	C1	KP257366	KP257300	KP257333	KP257399	2 ●
C. conv-fol complex	C2	KP257367	KP257301	KP257334	KP257400	2 ●
C. conv-fol complex	C3	KP257368	KP257302	KP257335	KP257425	2 ●
C. conv-fol complex	C4	KP257369	KP257303	KP257336	KP257427	2 ●
C. conv-fol complex	C5	KP257370	KP257304	KP257337	KP257426	2 ●
C. conv-fol complex	C6	KP257371	KP257305	KP257338	KP257428	2 ●
C. conv-fol complex	C7	KP257372	KP257306	KP257339	KP257401	2 ●
C. conv-fol complex	C8	KP257373	KP257307	KP257340	KP257402	2 ●
C. conv-fol complex	C9	KP257374	KP257308	KP257341	KP257403	2 ●
C. conv-fol complex	C10	KP257375	KP257309	KP257342	KP257404	2 ●
C. conv-fol complex	C11	KP257376	KP257310	KP257343	KP257405	2 ●
C. conv-fol complex	C12	KP257377	KP257311	KP257344	KP257406	2 ●
C. conv-fol complex	C13	KP257378	KP257312	KP257345	KP257407	2 ●
C. conv-fol complex	C14	KP257379	KP257313	KP257346	KP257408	3 ●
C. conv-fol complex	C15	KP257380	KP257314	KP257347	KP257409	3 ●
C. conv-fol complex	C16	KP257381	KP257315	KP257348	KP257410	6 ●
C. conv-fol complex	C17	KP257382	KP257316	KP257349	KP257411	6 ●
C. conv-fol complex	C18	KP257383	KP257317	KP257350	KP257412	6 ●
C. conv-fol complex	C19	KP257384	KP257318	KP257351	KP257413	11 ●
C. conv-fol complex	C20	KP257385	KP257319	KP257352	KP257414	1 □
C. conv-fol complex	C21	KP257386	KP257320	KP257353	KP257415	1 □
C. conv-fol complex	C22	KP257387	KP257321	KP257354	KP257416	1 □
C. conv-fol complex	C23	KP257388	KP257322	KP257355	KP257417	4 ■
C. conv-fol complex	C24	KP257389	KP257323	KP257356	KP257418	5 ■
C. conv-fol complex	C25	KP257390	KP257324	KP257357	KP257419	7 ▶
C. conv-fol complex	C26	KP257391	KP257325	KP257358	KP257420	8 ▶
C. conv-fol complex	C27	KP257392	KP257326	KP257359	KP257421	10 ■
C. rangiformis	C28	KP257393	KP257327	KP257360	KP257429	12 ○
C. rangiformis	C29	KP257394	KP257328	KP257361	KP257430	13 ○
C. rangiformis	C30	KP257395	KP257329	KP257362	KP257431	1 □
C. cervicornis s. str.	C31	KP257396	KP257330	KP257363	KP257422	9 ▶
C. cervicornis s. str.	C32	KP257397	KP257331	KP257364	KP257423	1 □
C. cervicornis s. str.	C33	KP257398	KP257332	KP257365	KP257424	1 □

C. convoluta-C. foliacea complex are indicated as *C. conv-fol* complex.

PCRs were performed in 50 µl using EmeraldAmp GT PCR Master Mix (Takara). The only user-supplied reagents that need to be added are template DNA, specific primers and water, allowing for improved reproducibility while minimizing the potential for contamination. Negative controls, without a DNA template, were included in every round of PCR amplification to ensure against false-positive results caused by contaminants in the reagents. The PCR programme for amplifications comprised an initial denaturation at 94°, 2 min, and 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. Amplifications were carried out on a 96-well SensoQuest labcycler (Progen Scientific). The PCR products were separated on 2 % agarose gels and purified using Real Clean Spin (Durviz). The amplified PCR products were sequenced with an ABI 3100 Genetic analyser using the ABI BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Sequence analyses. A multiple alignment of the newly determined fungal ITS rDNA (accession nos KP257399 to KP257424), algal ITS rDNA (KP257366 to KP257398), actin (KP257333 to KP257365) and LSU rDNA (KP257300 to KP257332) sequences and selected fungal ITS rDNA sequences described by Pino-Bodas *et al.* (2010) was built using MAFFT, version 6, applying the Q-INS-i strategy (Katoh *et al.*, 2002). The alignment of actin sequences was improved by eliminating the ambiguously aligned regions using the program Gblocks v. 0.91b (Castresana, 2000). The three loci were concatenated, yielding an alignment of 2036 characters. The final matrix contained 62 ITS rDNA, 62 actin and 34 LSU rDNA sequences. For each locus, the most appropriate substitution model was estimated using the Akaike information criterion (AIC) with PAUP/MrModeltest 1.0b (Nylander, 2004). This AIC-based model selection procedure selected the GTR + Γ model for the mycobiont ITS rDNA, and the three following models for the phycobiont datasets: (1) GTR + I + Γ for ITS rDNA, (2) GTR + Γ for the actin gene, and (3) HKY + I for LSU rDNA.

The phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al.*, 2012), carried out on partitioned datasets using the different substitution models selected by PAUP/MrModeltest 1.0b. All parameters were unlinked among partitions. Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for ten million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.0025. Finally, the burn-in values were determined using the 'sump' command.

Bootstrap analyses were performed by maximum-likelihood (ML) and weighted maximum-parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl, 2006) and PAUP*, version 4.0b10 (Swofford, 2002), respectively. ML analysis consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100 000). The analysis was performed on partitioned datasets using the different substitution models. The wMP bootstrapping (1000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean fit values for each character over all trees in the memory.

Haplotype network. To show the genetic diversity within the newly characterized lineages, we reconstructed the haplotype networks on the basis of MP analyses of all available sequences, including those selected from the GenBank database (Table 2), using the Haplotype Viewer (G. Ewing, available at www.cibiv.at/~greg/haploviewer).

Phycobiont ITS2 secondary structure. The coding regions, required for the basal stems in ITS2 secondary structures, were delimited by comparison between our sequences and known sequences of *Asterochloris* (Beiggi & Piercey-Normore, 2007). The stem–loop structures were folded using the ITS2DATABASE (Koetschan *et al.*, 2010). If more than one fold was produced, the final fold was based on comparisons with those previously published for *Asterochloris* (Beiggi & Piercey-Normore, 2007), maximizing the hydrogen bonding forming solid stems, and the largest negative Δg value (free energy).

RESULTS

Asterochloris mediterranea sp. nov. Barreno, Chiva, Moya et Škaloud (Figs 2, 3 and 4)

Description. Mature vegetative cells spherical in shape, but oval, pyriform and kidney shapes are not uncommon, 8.2–8.5 to 12–16.3 µm in diameter. Single central chloroplast

Table 2. GenBank fungal ITS rDNA sequences described by Pino-Bodas *et al.* (2010) included in the phylogenetic analysis and algal ITS rDNA (Pino-Bodas *et al.*, direct submission; Piercey-Normore & DePriest, 2001) included in the haplotype network analysis

Mycobiont	Collection	Fungal ITS	Algal ITS	Locality	Substrate
C. convoluta	MACB 90565	FM205886.1b	FM205732	Granada, Spain	Limestone ■
C. convoluta	MACB 90565	FM205886.1c	FM205730	Granada, Spain	Limestone ■
C. convoluta	MACB 91687	FM211899	FM205729	Guadalajara, Spain	Sandstone ▶
C. foliacea	MACB 95599	FM205914	FM205728	Trás-os-Montes, Portugal	Granite ▷
C. foliacea	MACB 90533	FM205897	FM205727	Guadalajara, Spain	Granite ▷
C. foliacea	MACB 90574	FM205895	FM205725	Tarragona, Spain	Granite ▷
C. foliacea	MACB 91639	FM205899	FM205724	Ávila, Spain	Quartzite □
C. convoluta	MACB 90440	FM205901	FM205723	Barcelona, Spain	Limestone ■
C. convoluta	MACB 90499	FM205900	FM205722	Navarra, Spain	Limestone ■
C. convoluta	MACB 90565	FM205886.1	FM205721	Granada, Spain	Limestone ■
C. foliacea	MACB 90503	FM205898	FM205720	Alto Alentejo, Portugal	Quartzite □
C. rangiformis	Frost-Olsen 5501	–	AF345435	Yugoslavia	–
Cladonia fimbriata	Gustavsson & Thollesson s.n.	–	AF345434	Gothenburg, Sweden	–

with margins extended into finger-like, divided lobes. Central pyrenoid, spherical or irregularly elongated, of *irregularis*-type. One nucleus with nucleolus. Cell wall thin, 0.3–0.5 μm wide, with flat local thickening and irregular secretory spaces. Asexual reproduction by 64–128 spherical aplanospores.

Type locality. Phycobiont of *C. convoluta-C. foliacea* complex, collected on gypsum soils in Fuentidueña de Tajo (Madrid, Spain), 40° 07′ 87″ N 03° 09′ 12″ W, altitude 571 m, upper mesomediterranean low dry (leg. Barreno, Chiva, Molins & Salvá 24 February 2012). The lichen specimen was deposited in herbarium PRC (no. 2939). Samples of the same population are in MA-Lich (no. 18201), VAL_Lich (no. 30278) and MAF-Lich (no. 19479).

Holotype. Cryopreserved cells of strain C6, deposited at the Culture Collection of Algae of the Charles University in Prague (CAUP), as item TYPE – H 1015.

Reference strains. CAUP H 1015 and E. Barreno's Lab in the Universitat de València (no. 131).

Molecular signatures. Hemi-CBCs in helix III (**C**-G : **U**-G; unique) and (**G**-G : **C**-G) of the ITS2 as compared with the ITS2 rRNA secondary transcripts of *Asterochloris phycobiontica*.

Etymology. *Asterochloris mediterranea* (me.di.ter.ra′ne.a.).

Etymology: L. fem. adj. *mediterranea* midland, inland and, in late Latin, used to refer to the Mediterranean Sea or bioclimatic types (Rivas-Martínez & Rivas-Sáenz, 2009).

The specific epithet refers to the wide but not exclusive Mediterranean distribution and the relative abundance of several *Cladonia* mycobionts, which are up to now the most frequent hosts of this phycobiont.

Distribution. So far only known in the Iberian Peninsula, former Yugoslavia, Sweden and the Canary Islands.

Ecology. At the time of writing it has been found in symbiosis with *C. convoluta-C. foliacea* complex, *C. rangiformis*, *Cladonia fimbriata* and *C. cervicornis* s. str. lichen thalli growing in a wide variety of habitats, from lowlands to Mediterranean mountains, and on soils derived from very different types of rocks (gypsum, limestones, quartzites, granites, sandstones, volcanic).

Morphological and ultrastructural characterization

The squamules analysed in this study showed a strongly fissured and cracked surface (Fig. 2a, b), similar to the features previously noticed by Osyczka & Rola (2013), who found a full range of surface roughness under SEM in different species of the genus *Cladonia* growing in non-arid or dry habitats.

In this study, LM, SEM and TEM were used to characterize the structure and ultrastructure of the cells of *Asterochloris mediterranea* sp. nov. found in *C. convoluta-C. foliacea* complex, *C. rangiformis* and *C. cervicornis* s. str. (Figs 2 and 3). The observations were made on samples C6 and C16, and no differences were found between them. The phycobionts were usually located in close contact with the hyphae in the phycobiont layer; the interactions between photobiont cells and fungal hyphae were the 'simple' type (Honegger, 1986), without invaginations or haustoria (Fig. 2a, b, c). Mature vegetative cells were mostly spherical, but oval, pyriform and kidney shapes were also common, 8.2–8.5 to 12–16.3 μm in diameter. The cell wall exhibited a variable thickness, ranging from 0.3 to 0.5 μm, with flat local thickening and irregular secretory spaces giving a characteristic appearance to the young and mature vegetative cells. Both in thallus and culture, the cells showed a large, axial and lobed chloroplast with margins extended into finger-like lobes in the periphery characteristic of the genus *Asterochloris* (Fig. 2c, d). The thylakoid membranes were grouped in stacks shaped by 4–5 membranes, similar to the grana in vascular plants (Fig. 3b, c, e, f). A large central portion of the chloroplast was taken up by the pyrenoid (Figs 2e, f and 3a, c, d). Usually, there was only one pyrenoid per cell, although in some cases two could appear (Fig. 3d). This pyrenoid might be spherical or irregularly elongated (Fig. 3a, c), with a small number of thin, arched thylakoid tubules invaginating within the matrix. Numerous pyrenoglobuli (80–100 nm in diameter) were connected with these tubules (Fig. 3b). This ultrastructure fits into the *irregularis*-type (Friedl, 1989). Spherical non-electron-dense vesicles appeared throughout the cytoplasm and were especially numerous at the periphery (Figs 2e, f and 3a). Also, some small mitochondria were observed (Fig. 3e). Most of the cells presented numerous ribosomes in the cytoplasm (Fig. 3e, f) and showed a clear and apparent nucleus with nucleolus (Figs 2f and 3d). The plasmatic membrane showed invaginations in many areas connected with secretory vesicles (Fig. 3e). Secretory spaces were irregular in thickness and myelin-like bodies could be seen in some cases (Fig. 3e). Two types of myelin-like bodies were detected, the plasmalemmasomes in the secretory spaces (Fig. 3e) outlined by evagination of the plasmalemma as well as by fusion of vesicles, and lomasomes (Fig. S1) associated with the rough endoplasmic reticulum (Marchant & Robards, 1968; Robards, 1968). Aplanospores by 64–128 in the sporocysts were spherical (Fig. 2d).

Chloroplast morphology of isolated lichen phycobionts

Once unialgal cultures were obtained as previously described, confocal reconstructions showed that the majority of the cell volume was occupied by the chloroplast (Fig. 4). In young cells, the chloroplast was central, axial, with simple lobes spreading towards the cell periphery (Fig. 4a). Mature vegetative cells could possess a similarly formed, shallowly lobed plastid with simple lobes. However, the lobes were

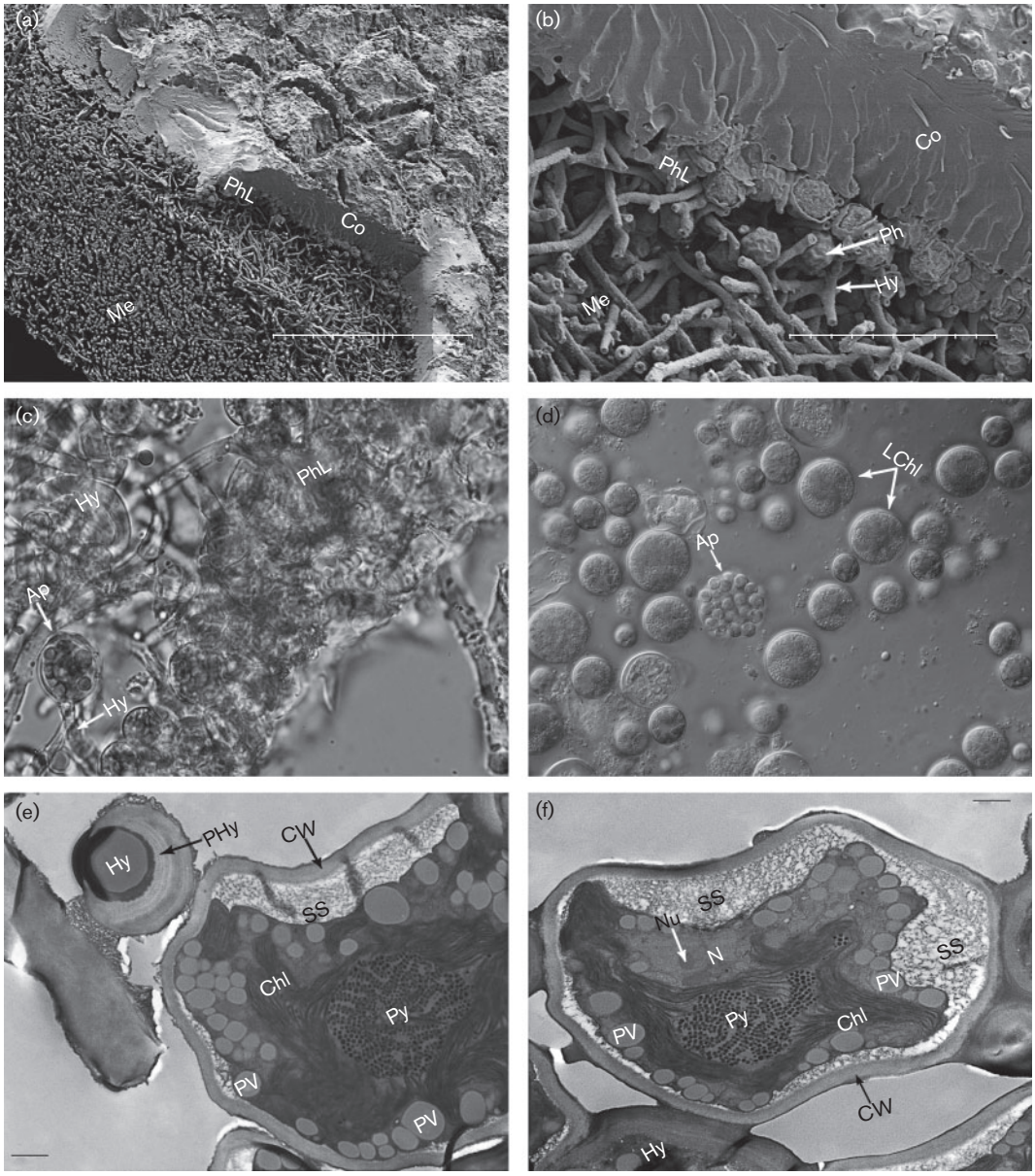

Fig. 2. Ultrasculpture architecture of *C. convoluta*-*C. foliacea* complex thalli C16 by SEM. (a) Transversal section of the primary thallus with upper cortex (Co), phycobiont layer (PhL) and medulla (Me); bar, 200 μm. (b) Detail of the phycobiont layer (PhL) showing the interaction between phycobiont (Ph) and hypha (Hy) of the type 'simple' (Honegger, 1986); bar, 40 μm. (c) Cross section of the thallus by LM (sample C16, fresh). Phycobiont layer (PhL) showing asexual reproduction by aplanospore (Ap). (d) Isolated *Asterochloris*-type phycobiont showing aplanospore (Ap) and characteristic 'lobed' *Asterochloris* chloroplast (LChl). Cross section of C16 thallus by TEM (e) and (f) *Asterochloris mediterranea* sp. nov. phycobiont inside thallus. Bars, 1 μm. PHy, Peripherial hypha; CW, cell wall; SS, secretory space; Chl, chloroplast; Py, pyrenoid; PV, peripheral vesicles; N, nucleus; Nu, nucleolus.

often branched at their ends, so the chloroplast margin was extended into finger-like, divided lobes (Fig. 4b, c). Rarely, the chloroplast assumed a parietal position, having short, simple lobes. In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transformed into the parietal type with smooth, never lobed, margins. After a short time, it began to divide into numerous parts in preparation for asexual reproduction (Fig. 4d).

Phycobiont phylogenetic analysis

At the time of writing, apart from sequences obtained in this study, a total of 19 nrITS phycobiont sequences from *C.*

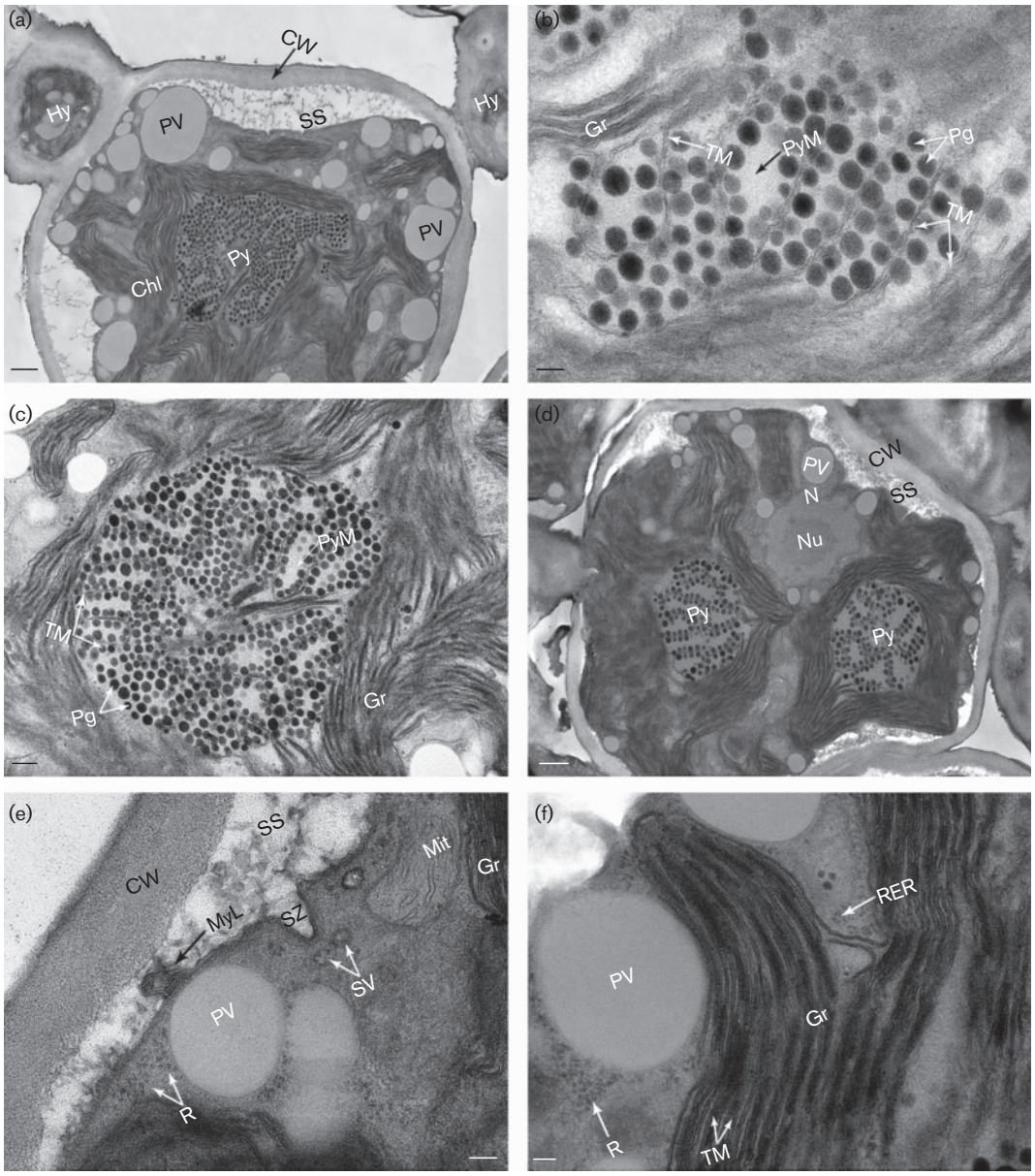

Fig. 3. *C. convoluta*-*C. foliacea* complex. Cross section of C16 thalli by TEM. (a) *Asterochloris mediterranea* sp. nov. phycobiont inside thallus; bar 1 μm. (b) and (c) Detail of a pyrenoid (Py), pyrenoglobuli (Pg) associated with the pyrenoid matrix (PyM), grana (Gr) and thylakoid membrane (TM); bars 0.2 μm and 0.5 μm, respectively. (d) Phycobiont showing chloroplasts in duplication phase with two pyrenoids (Py), peripheral vesicles (PV), nucleus (N), nucleolus (Nu), cell wall (CW), secretory space (SS); bar 1 μm. (e) Detail showing secretory complex: myelin-like bodies (MyL), peripheral vesicles (PV), mitochondria (Mit), ribosome (R), secretory space (SS), secretory vesicle (SV), secretion zone (SZ) and rough endoplasmic reticulum (RER); bar 0.2 μm. (f) Detail of chloroplast showing peripheral vesicles (PV), ribosome (R), thylakoid membrane (TM), rough endoplasmic reticulum (RER) and grana (Gr). Bar 0.2 μm

convoluta, *C. foliacea*, *C. rangiformis* and *C. cervicornis* subsp. *verticillata* were available in GenBank (Piercey-Normore & DePriest, 2001; Beiggi & Piercey-Normore, 2007; Bačkor *et al.*, 2010; Pino-Bodas *et al.*, 2010). Only 11 sequences from the *C. convoluta*-*C. foliacea* complex, submitted by Pino-Bodas (Table 2), fitted with those of *Asterochloris mediterranea*. All of them incorporated information about mycobiont nrITS and substrate. In addition, two sequences (AF345434 and AF345435) from *C. fimbriata* and *C. rangiformis* produced by Piercey-Normore & DePriest (2001) also fitted with those of *A. mediterranea*, but without any specific information concerning the habitat, location or nrITS sequence of the mycobiont. We included these sequences (Table 2) in the haplotype parsimony networks analysis, but they were excluded from the phycobiont phylogeny analysis due to the lack of actin and LSU rDNA information.

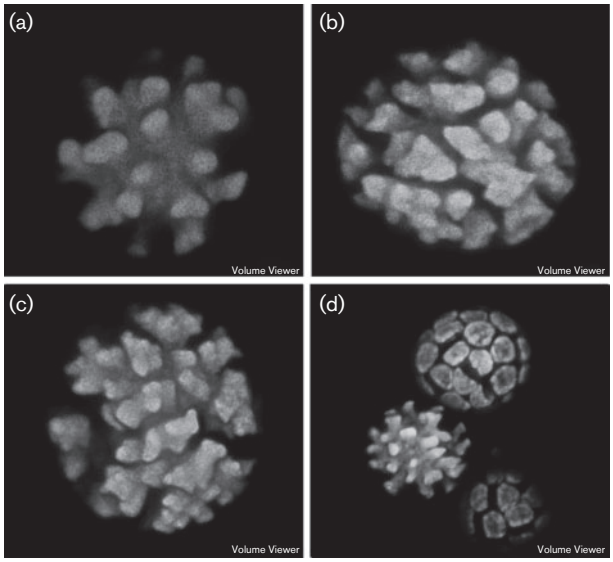

Fig. 4. Confocal microscopy of chloroplast structures in isolated lichen phycobionts (*A. mediterranea* sp. nov.). (a) A young cell with simple lobes, (b) a mature vegetative cell showing shallowly lobed plastid with simple lobes, (c) a mature vegetative cell with finger-like divided lobes, (d) two globular aplanosporangia with a large number of aplanospores.

The concatenated Bayesian analysis of the new phycobiont ITS rDNA, actin and LSU rDNA dataset revealed the existence of more than 20 well-resolved lineages within the genus *Asterochloris* (Fig. 5). The relationships among the lineages correspond well with the phylogeny presented by Škaloud & Peksa (2010) and Škaloud *et al.* (2015), including the presence of three moderately to well-supported major clades, A, B and C. The 13 previously described species (*A. erici*, *A. excentrica*, *A. glomerata*, *A. irregularis*, *A. italiana*, *A. magna*, *A. phycobiontic*a, *A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprarii*, *A. lobophora* and *A. woessiae*) formed well-recognized, distinct lineages. All phycobionts investigated in this study formed a distinct, statistically well-supported lineage, inferred within clade C, in relation to the lineage represented by a single *Lepraria* phycobiont Nelsen 2585.

Genetic relationships of sequences of *A. mediterranea* from samples C1 to C33 (Table 1) and the sequences selected in Table 2 were analysed by statistical parsimony networks of the ITS rDNA showing minimum differences. All the haplotypes were included in one linked network pointing out a single taxon. Six haplotypes of *A. mediterranea* were found in the *C. convoluta-C. foliacea* complex, and two more haplotypes in the three *Cladonia* taxa studied here (Fig. 6a). Haplotype 7 (AF345434) and Haplotype 9 (AF345435) were inferred from GenBank. In lichens growing at localities with Miocene gypsum bedrock, only four haplotypes were detected (Fig. 6b), whereas another four haplotypes appeared randomly in the remaining types of substrates.

ITS2 secondary structure

A common overall organization of the ITS2 secondary structure could be identified in *A. mediterranea* (Fig. 7). The ITS2 secondary structure possessed conserved motifs among green algae (Mai & Coleman, 1997), i.e. four-fingered hand (helix I-IV), a pyrimidine-pyrimidine mismatch in helix II, and a conserved sequence of UGGU on the 5′ side of helix III (Fig. 7). The ITS2 secondary structures were compared first among the *A. mediterranea* genotypes found in this study to check the occurrence of compensatory base changes (CBCs: nucleotide changes at both sides of paired bases) and hemi-CBCs (changes at only one side of a nucleotide pair, but still preserving pairing) according to Coleman (2003). One insertion, one single base change and one hemi-CBC at positions 18, 80 and 88 were identified; these changes were not previously identified by Škaloud & Peksa (2010).

The ITS2 secondary structure of *A. mediterranea* was also compared with the previously published structures of *Asterochloris* lineages 1–16 (Škaloud & Peksa, 2010). In total, two single base changes at positions 35 and 37, and one hemi-CBC at position 91, were newly identified.

Mycobiont phylogenetic analysis

To validate the correct lichen identifications, 33 samples were analysed, all of them with the corresponding algal sequences (Table 1). We also included 11 mycobiont sequences selected from those described by Pino-Bodas *et al.* (2010) in which the phycobiont partner sequences coincided with those of *A. mediterranea* (Table 2).

The aligned fungal ITS was 481 bp long, including ITS1, 5.8S rDNA and partial ITS2, with 91 variable characters of which 64 were parsimony-informative. We resolved 20 fungal ITS genotypes: 14 in the *C. convoluta-C. foliacea* complex, three in *C. rangiformis* and two in *C cervicornis* s. str. (Fig. 8). BLAST searches produced significant matches with other fungal accessions of species of the genus *Cladonia* described by Pino-Bodas *et al.* (2010, 2013a) and Stenroos *et al.* (2002b). Phylogenetic analysis including mycobiont ITS sequences from Pino-Bodas *et al.* (2010) selected in this study, showed three well-supported clades corresponding with the *C. convoluta-C. foliacea* complex, *C. rangiformis* and *C. cervicornis* s. str. (Fig. 8) as we previously determined.

DISCUSSION

The present study contributes to the understanding of the symbiont microalgae of *Cladonia* lichens providing new insights into the hidden phycobiont diversity found in the genus *Asterochloris* (Tschermak-Woess, 1980; Bačkor *et al.*, 2010; Škaloud & Peksa, 2010; Peksa & Škaloud, 2011; Škaloud *et al.*, 2015). Both morphological and molecular analyses pointed out the presence of a previously unknown taxon described here as *Asterochloris mediterranea* sp. nov.

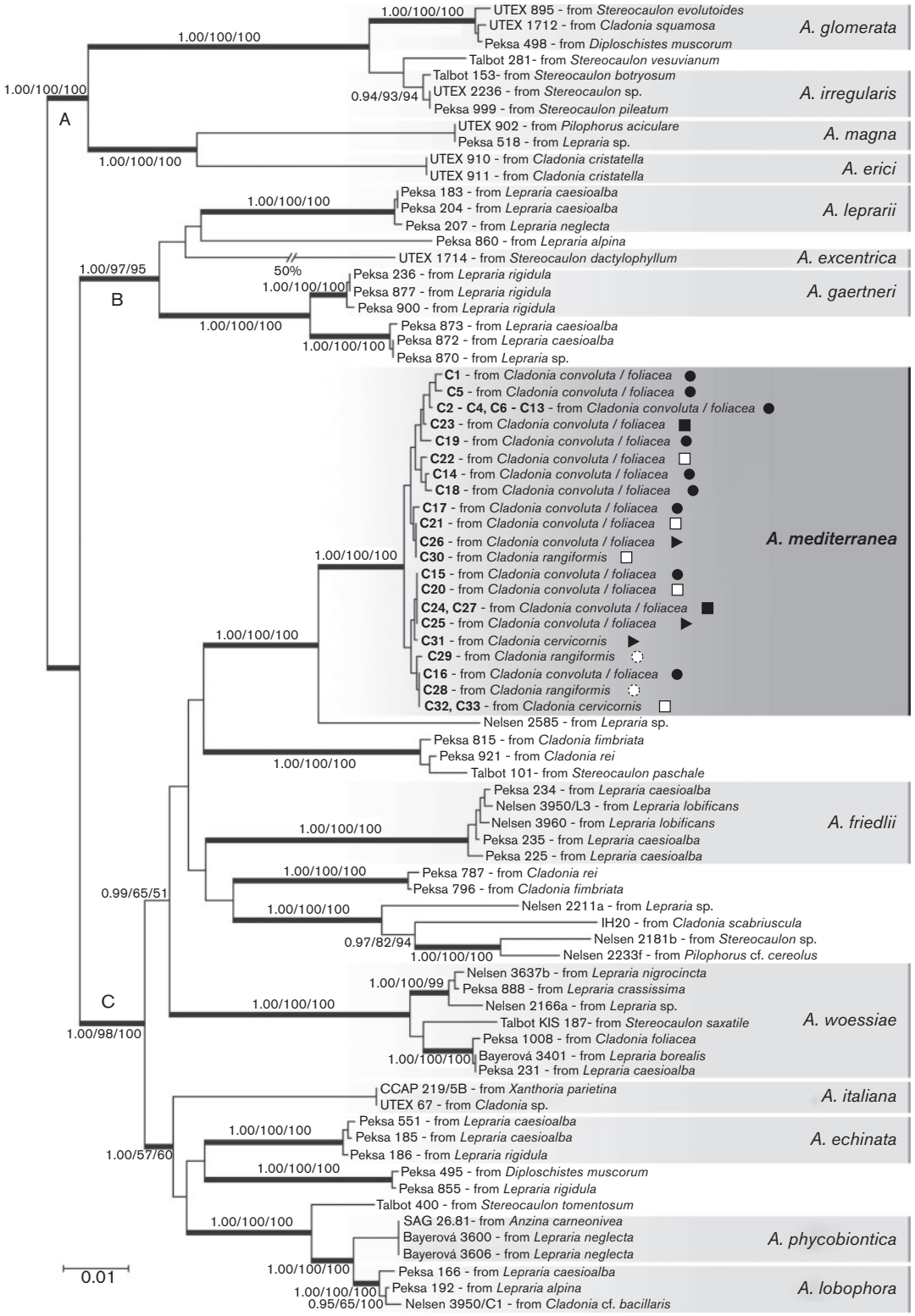

Fig. 5. Unrooted BI analysis based on the combined ITS rDNA + LSU rDNA + actin dataset using the GTR + I + Γ model for ITS rDNA, HKY + I model for LSU rDNA and GTR + Γ model for the actin gene. Values at nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), ML bootstrap (middle) and MP bootstrap (right). Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are given in bold type with the type of substrate indicated by symbols: quartzites, siliceous (white square □), limestones, calcareous (black square ■), sandstones, siliceous (black triangle ►), Miocene gypsum (black circle ●) and volcanic (white discontinuous circle

◌). Authentic strains of the genus *Asterochloris* are marked in grey and the newly defined *A. mediterranea* is given in bold type. Strain affiliation to three major clades (A–C) is indicated. Bar, estimated number of substitutions per site.

Barreno, Chiva, Moya et Škaloud linked to the thalli of several members of the genus *Cladonia* which are widely but not exclusively distributed in Mediterranean and Canarian ecosystems.

This genus was described by Tschermak-Woess (1980) who separated it from the genus *Trebouxia* on the basis of chloroplast morphology, and later this was supported by more recent confocal and molecular analyses (del Campo *et al.* 2010a; Peksa & Škaloud 2008; Škaloud & Peksa, 2008a, b, 2010; Škaloud *et al.*, 2015). In this work, additional features based on TEM observations and on symbiotic state are proposed for the characterization of species of the genus *Asterochloris*. The plastid molecular marker LSU rDNA (23S) recommended by del Campo *et al.* (2010a) was incorporated to build the algal phylogeny, together with the traditionally used nuclear ITS rDNA and actin intron markers (Škaloud & Peksa, 2010). The correct

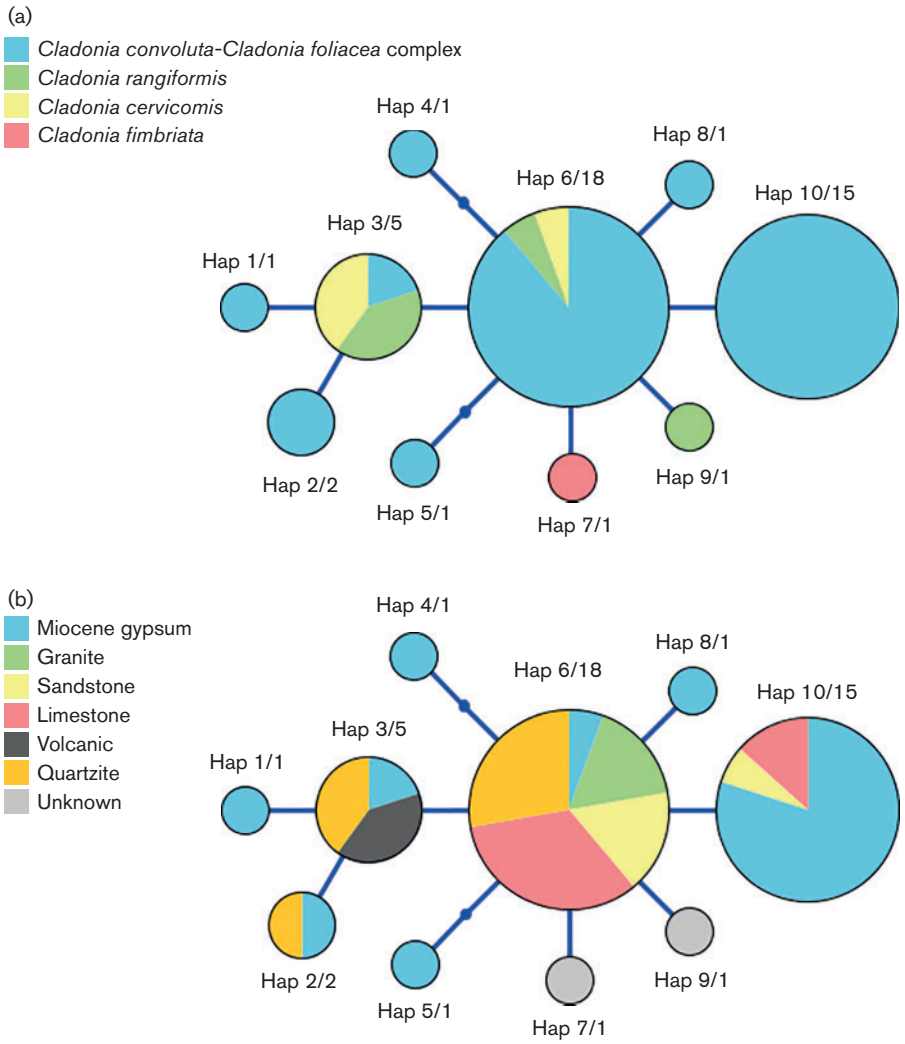

Fig. 6. Statistical parsimony networks obtained for the ITS rDNA *A. mediterranea* haplotypes found in this study including all available sequences selected from the GenBank database. Size of the circles is proportional to the number of samples sharing that haplotype. Number of samples found sharing that haplotype is given after the haplotype number. Colours in the upper network (a) denote taxa: *C. convoluta-C. foliacea* complex blue, *C. rangiformis* green, *C. fimbriata* pink and *C. cervicornis* s. str pale yellow. Colours in the lower network (b) denote type of substrate: Miocene gypsum blue, granite green, sandstone pale yellow, limestone pink, volcanic dark grey, quartzite orange, unknown grey.

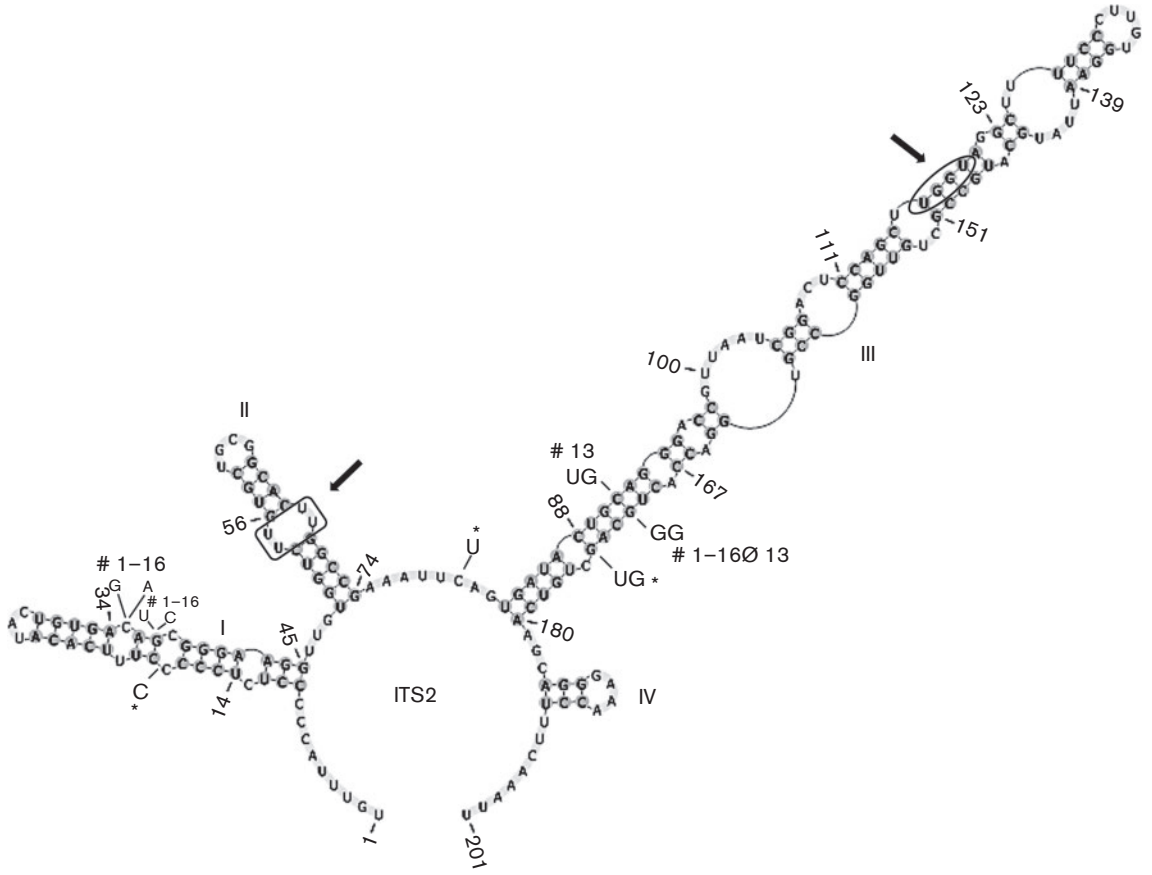

Fig. 7. Predicted secondary structure of the ITS2 transcript of *A. mediterranea.* Base changes between *A. mediterranea* genotypes found in this study are indicated with an asterisk; one single base change, one insertion and one hemi-compensatory base change (CBC). New base changes between *A. mediterranea* and 16 *Asterochloris* lineages defined by Škaloud & Peksa (2010) are also indicated; two single base changes and one hemi-CBC. The numbers next to the number sign (#1–16) specify the *Asterochloris* clades in which the base changes occurred. The highly conserved U-U in helix II and UGGU motif in helix III (both arrows) are highlighted (Schultz *et al.*, 2005).

identification of the fungal partners was corroborated by the nrITS barcode.

Cellular characteristics in species of the genus *Asterochloris* have been mostly described from *in vitro* cultures by CM and/or LM techniques. Algae undergo a variety of structural, physiological, and biochemical modifications as a result of lichenization (Galun, 1988; Friedl & Büdel, 2008); therefore, it seems likewise necessary to know the symbiotic state. TEM ultrastructural observations in symbiosis indicated that in mature cells one large central pyrenoid was always present; sometimes two appeared when the chloroplast was in the duplication phase (Fig. 3d). The pyrenoid ultrastructure fits with the *irregularis*-type (Fig. 3c) (Friedl, 1989) observed in most species of the genus *Asterochloris* delimited by Škaloud & Peksa (2010) (i.e. *A. excentrica*, *A. glomerata*, *A. irregularis*, *A. italiana* and *A. pyriformis*).

Škaloud & Peksa (2008a) proposed that chloroplast morphology could be considered as an important morphological marker for delimitation of species of the genus *Asterochloris* under culture conditions. The shallowly lobed plastid with either simple or finger-like lobes, observed in *A. mediterranea*, also occurred in *A. excentrica*, *A. irregularis* and *A. friedlii* (Table S2) (Škaloud & Peksa, 2008b). Nevertheless, this study has illustrated that CM and TEM techniques should be complementary in the characterization of the chloroplast morphologies (Figs 2e, f and 4) due to the differences in thylakoid arrangements (Fig. 3b, f), as proposed by del Campo *et al.* (2010b) and Casano *et al.* (2011) for the taxa *Trebouxia* TR1 and *Trebouxia* TR9. The frequent presence of evident nuclei with nucleoli and high amounts of ribosomes could be related to intense metabolic activity of the cells (Boisvert *et al.*, 2007). In addition, the different myelin-like bodies (plasmalemmasomes, lomasomes) in secretory spaces and endoplasmic reticulum highlight the strong cell activity which is required to remove the excess of membranes (Marchant & Robards, 1968; Robards, 1968). The variable shape of mature cells (spherical to pyriform or kidney-shaped) together with the irregular secretory spaces and the flat and

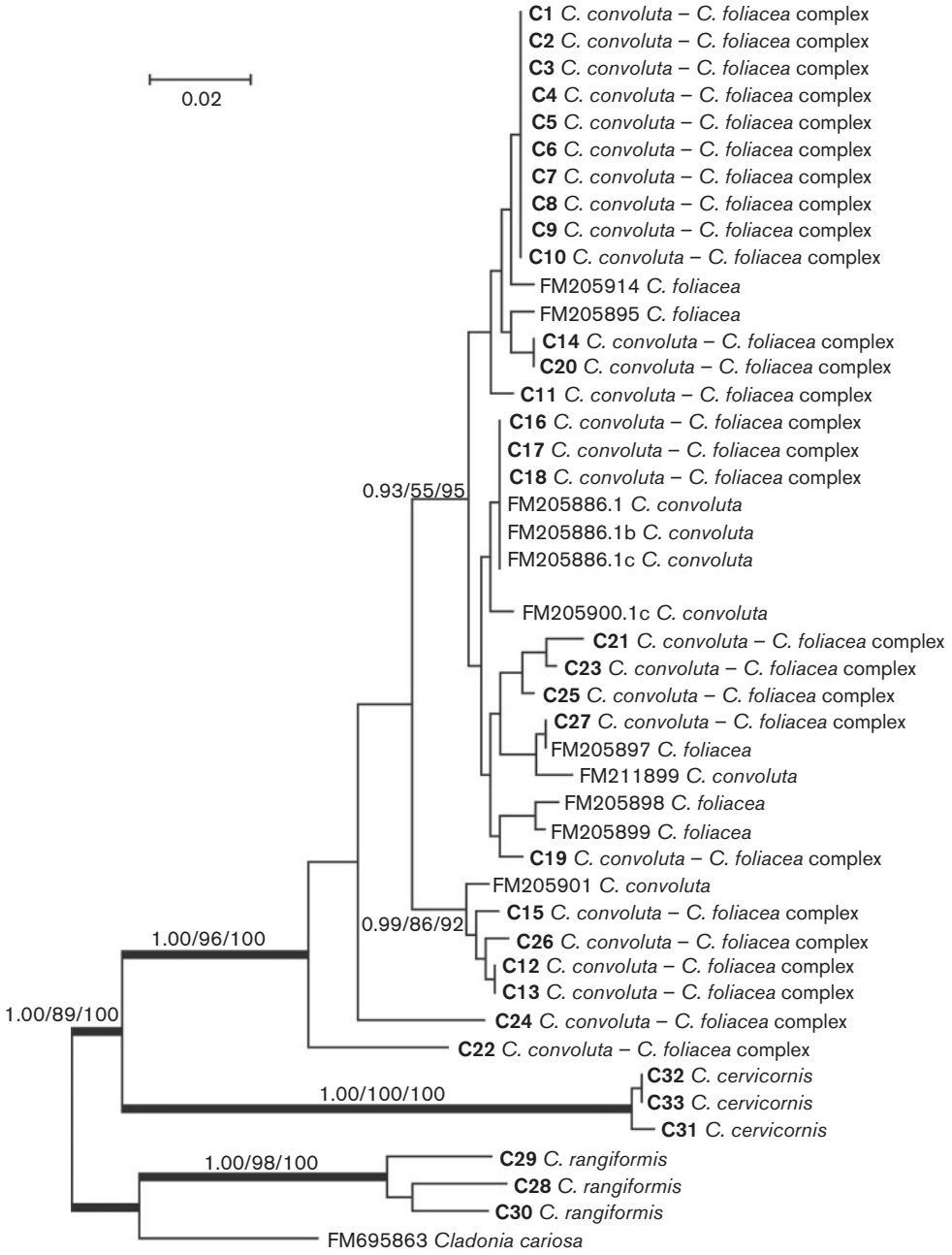

Fig. 8. The BI analysis of mycobiont ITS rDNA using the GTR + Γ model. Values at nodes indicate statistical support estimated by three methods – MrBayes posterior node probability (left), ML bootstrap (middle) and MP bootstrap (right). Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are given in bold type. Accession numbers of sequences selected from Pino-Bodas *et al.* (2010) accompany each species name. Bar, estimated number of substitutions per site.

delimited thickening of the cell wall could be useful features to help distinguish species of the genus *Asterochloris* from those of the genus *Trebouxia* (data not published).

Species delimitation methods based on single-locus data rely on the assumption that a single gene genealogy is sufficient to illustrate species phylogeny. In *Asterochloris* algae, evolutionary inferences based on two molecular markers (ITS rDNA and actin sequences) revealed extensive diversity of this algal genus (Škaloud & Peksa, 2010; Škaloud *et al.*, 2015). In this work, evolutionary inferences based on multiple loci, both nuclear (nrITS and actin) and chloroplast-encoded (LSU rRNA), helped us to reinforce the *Asterochloris*

phylogeny suggested by Škaloud & Peksa (2010) and revealed a new well-supported clade (100 % MrBayes/ML/MP) here described as *Asterochloris mediterranea* sp. nov.

Parsimony networks using our samples C1 to C33 and the 13 GenBank sequences showed minimum intraspecific genetic variation among all the haplotypes detected. In addition, we were not able to find any relationship between the haplotype distributions, neither in the types of substrata nor the *Cladonia* taxa studied here, although Peksa & Škaloud (2011) demonstrated clear ecological preferences among the majority *of Asterochlor*is lineages.

Putative models of secondary structures have been characterized for the ITS regions of rRNA (Coleman *et al.*, 1998; Joseph *et al.*, 1999; Lalev & Nazar1998, 1999; Mai & Coleman, 1997). The maintenance of these structures is important for the proper functioning of the rRNA (Coleman, 2003) and, therefore, ITS rRNA transcripts in *Asterochloris* have been used as an additional attribute to delimit species boundaries (Beiggi & Piercey-Normore, 2007; Škaloud & Peksa, 2010; Škaloudová & Škaloud, 2013). However, it has recently been suggested that differences in the ITS rRNA secondary structures are often not diagnostic at the species level in green algae (Caisová *et al.*, 2011; Škaloud & Rindi, 2013). Therefore, we would rather consider the presence of nucleotide substitutions in stem regions of the ITS2 rRNA transcript as an attribute of elapsed evolutionary time, indicating that sufficient time has passed to produce a speciation event. Comparing *A. mediterranea* ITS rRNA secondary structure with the *Asterochloris* lineages described by Škaloud & Peksa (2010), low genetic variation was found in ITS rDNA gene regions, which correlates with the absence of CBCs.

In the genus *Cladonia*, identification of the fungal partners is often problematic and BLAST searches have showed high failure rates (Kelly *et al.*, 2011), thus the DNA barcoding for fungi (nrITS sequences) proposed by Schoch *et al.* (2012) was used. Even using the barcoding molecular marker, some lichen taxa still remain problematic (Pino-Bodas *et al.*, 2013a). Several studies have shown that the nrITS region provides a poor resolution for certain species in the genus *Cladonia* (Fontaine *et al.*, 2010; Kotelko & Piercey-Normore, 2010; Pino-Bodas *et al.*, 2010, 2013b; Steinová *et al.*, 2013). Specifically, *C. convoluta* (Lam.) Anders and *C. foliacea* (Huds.) Willd. the currently available data hindered the delimitation of two monophyletic groups (Pino-Bodas *et al.*, 2010). Only three species of the genus *Cladonia* were separated as independent monophyletic groups, with *C. convoluta* and *C. foliacea* joined together in the same clade, confirming the results found by Pino-Bodas *et al.* (2010). Although, the case of *C. cervicornis* complex was clearly solved by Pino-Bodas *et al.* (2013b).

In summary, the genus *Asterochloris* is the preferential phycobiont in *Cladonia*, *Lepraria* and *Stereocaulon* lichens, and the diversity of this algal genus needs to be deeply explored in different mycobionts, areas and habitats. The combination of several nuclear and plastid molecular markers as well as ultrastructural (TEM and CM) techniques both in culture and in the symbiotic state should be utilized. *Asterochloris mediterranea* might exemplify this assertion.

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