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.

### 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 (phycobiont) 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 undergo 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 (Myllös et al., 2003; Kotelo & 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 Cladoniinae (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 (Rídká 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 unicellular 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 % OsO₄ 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). These 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

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**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-dimensional reconstruction of their morphology. The chloroplast reconstructions were produced by the ImageJ 1.34p program (Abramoff 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)

<table>
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<tr>
<th>Taxon</th>
<th>Code</th>
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<th>Locality &amp; type of substrate</th>
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<td></td>
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<td></td>
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<td>C33</td>
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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 °C, 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 SonHoQuest 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 analysis 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 alignment of 2036 characters. The final matrix contained 62 ITS rDNA sequences described by Pino-Bodas et al. (2010). The phylogenetic trees were inferred by Bayesian inference (BI) using MrBays 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 two hot chains. 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 (genethreshfortopoterm 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 recursively 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 (Beippi & Piercey-Normore, 2007). The stem–loop structures were folded using the ITS2DATEBASE (Koetschan et al., 2010). If more than one fold was produced, the final fold was based on comparisons with those previously published for Asterochloris (Beippi & 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 in diameter. Single central chloroplast

<table>
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<th>Mycobiont</th>
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<td>–</td>
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</table>
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 plasmalemmosomes 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
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.*
convoluta, C. foliacea, C. rangiformis and C. cervicornis subsp. verticillata were available in GenBank (Piercey-Normore & DePriest, 2001; Beiggi & Piercey-Normore, 2007; Băckö 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. 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.
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. phycobiontica, A. echinata, A. friedlilii, 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 symbiotic microalgae of Cladonia lichens providing new insights into the hidden phycobiont diversity found in the genus Asterochloris (Tschermak-Woess, 1980; Bacˇkor 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+I+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 -).
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

![Diagram](image)

**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.

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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. 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).
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* 

![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.](image-url)
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 *Asterochloris* 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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