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Standard Paper

Symbiont composition of the basidiolichen *Lichenomphalia meridionalis* varies with altitude in the Iberian Peninsula

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Abstract

Basidiolichens are generally poorly researched because of the very small number of species and their restriction to special niches. *Lichenomphalia* basidiolichens grow in considerable quantities in arctic and alpine habitats but they are inadequately studied in these habitats in Mediterranean areas. Based on morphological and phylogenetic analyses, we identified the different symbionts of *L. meridionalis*, collected in localities in Spain at altitudes ranging from 533 to 2200 m above sea level. The present study provides the first molecular data available for *L. meridionalis*. We found that a microindel of six bp within the nrITS2 could help to discriminate *L. meridionalis* from other species of the genus. Molecular analyses revealed the existence of two different green algal strains, both belonging to *Coccomyxa subellipsoidea*, a species shared with other *Lichenomphalia* lichens. Notably, the two chlorobiont strains associated with *L. meridionalis* were differentially distributed according to altitude, and samples having one of the two strains consistently also included cyanobacteria.

Key words: arctic-alpine, *Coccomyxa*, cyanobacteria, lichen, Mediterranean, symbiosis

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Introduction

Lichens are complex systems involving symbiotic associations of at least two very different organisms, a heterotrophic fungus (mycobiont) and a photobiont, a unicellular to filamentous green algae (chlorobiont; Chapman & Margulis 1998) and/or a cyanobacteria (cyanobiont). Lichens are found in almost all terrestrial habitats and geographical areas, demonstrating the successful nature of the symbiosis. Most mycobionts (>99%) are members of the Ascomycota, whereas lichen-forming Basidiomycota represent less than 0.9% of all lichenized fungi (Lücking *et al.* 2017*a*). In recent years, the classification of lichen-forming Basidiomycota has been continuously and substantially modified based on molecular data. These modifications include the introduction of new taxa including orders, families, subfamilies, tribes and genera (Redhead *et al.* 2002; Ertz *et al.* 2008; Lawrey *et al.* 2009; Dal Forno *et al.* 2013; Hodkinson *et al.* 2014; Lücking *et al.* 2017*a*).

Hygrophoraceae is one of 20 fungal families with the largest number of lichenized species (Lücking *et al.* 2014, 2017*a*; Dal Forno *et al.* 2016). This family includes at least 23 genera according to Lodge *et al.* (2014), six of which are lichenized, namely *Acantholichen*, *Cora*, *Corella*, *Cyphellostereum*, *Dictyonema* and *Lichenomphalia*. While the first five genera are mainly tropical to tropical montane, *Lichenomphalia* species have traditionally been considered to have an arctic-alpine distribution, with the exception of *L. umbellifera* which is also common in northern forests (Redhead & Kuyper 1987; Kranner & Lutzoni 1999; Redhead *et al.* 2002; Lawrey *et al.* 2009; Geml *et al.* 2012).

Lichenomphalia species have frequently been reported from central and northern Europe, but only occasionally from southern Europe (Nimis 1993; Suppan *et al.* 2000). In the Iberian Peninsula, at least four *Lichenomphalia* species have been found (Barrasa & Rico 2001): *L. hudsoniana*, *L. meridionalis*, *L. velutina* and *L. umbellifera* (previously known as *Omphalina ericetorum*). All have a thallus of hyphal globules (*Botrydina*-type) except *L. hudsoniana*, which has a squamulose vegetative thallus (*Coriscium*-type) (Redhead & Kuyper 1987). The four species present different ecogeographical adaptations to specific regions: *L. meridionalis* seems to be restricted to the Mediterranean region, inhabiting more or less humid sites on the supra-Mediterranean belt, whereas *L. hudsoniana* is known only from the montane belt of the Eurosiberian region and the other two species, *L. umbellifera* and *L. velutina*, are widespread.

Lichenomphalia meridionalis (Contu & La Rocca) P.-A. Moreau & Courtec. has been reported only in the Mediterranean region, in Sardinia (Italy) (Contu & La Rocca 1999), where it was originally found, and Spain (Barrasa & Rico 2001). The aim of the present study is to increase our knowledge of the poorly studied Mediterranean basidiolichens by answering two questions: 1) is *L. meridionalis* distinguishable from other closely related *Lichenomphalia* species on the basis of molecular data, and 2) are the chlorobionts of *L. meridionalis* different from those of other *Lichenomphalia* species? Microscopic observations and molecular markers were used to investigate symbiont composition in specimens collected at different altitudes in several localities on the Iberian Peninsula.

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Table 1. Samples of *Lichenomphalia meridionalis* with details of voucher information (AH = Alcalá Herbarium), origin in Spain (including altitude (m above sea level)) and collection dates.

Isolate	Voucher information	Collection date	Origin (Spain)	Altitude (m)
Lms 1	AH: 23291	16-07-1997	Ibón Machimaña, Panticosa, Huesca	2200
Lms 2	AH: 23331	13-08-1997	Ibón de Culibillas, Formigal, Huesca	1800
Lms 3	AH: 33747	26-10-2002	Pt° de la Quesera, Riaza, Segovia	1757
Lms 4	AH: 32757	16-10-2003	La Pinilla, Riaza, Segovia	1443
Lms 5	AH: 39235	29-12-2000	Molino de Cantalojas, Guadalajara	1342
Lms 6	AH: 37721	15-05-2004	San Martín del Pimpollar, Ávila	1335
Lms 7	AH: 27351	09-12-2000	La Pradera, Riaza, Segovia	1190
Lms 8	AH: 2817	17-11-1982	Montejo de la Sierra, Madrid	1148
Lms 9	AH: 25359	29-12-1999	Fresno de Cantespino, Segovia	1050
Lms 10	AH: 25152	16-01-1999	Fresno de Cantespino, Segovia	1050
Lms 11	AH: 32760	11-10-2003	Fresno de Cantespino, Segovia	1050
Lms 12	AH: 39243	01-11-2015	Fresno de Cantespino, Segovia	1050
Lms 13	AH: 37724	11-12-2003	Tamajón, Guadalajara	1026
Lms 14	AH: 13194	25-10-1990	Tamajón, Guadalajara	1026
Lms 15	AH: 39237	19-11-1996	Almoróx, Toledo	533

Materials and Methods

Taxon sampling and datasets

We analyzed *L. meridionalis* samples, including both vegetative thalli and basidiomata, collected in natural habitats from nine localities in Spain at altitudes ranging from 533 to 2200 m (Table 1). Specimens are preserved in the University of Alcalá Herbarium (AH).

Morphological examination of 'in thallus' and isolated lichen chlorobionts

We examined a total of fifteen specimens of *L. meridionalis* thalli. A macroscopic photograph of basidiomata was taken using an Olympus E-510 camera. Chlorobionts of *L. meridionalis* were isolated from a fresh lichen thallus (sample Lms12) according to Gasulla *et al.* (2010). The isolated chlorobionts were axenically cultured on small nylon square membranes in semi-solid Bold 3N medium (Bold & Parker 1962). They were incubated in a growth chamber maintained at a continuous temperature of 15 °C, with light/dark cycles of 14/10 h. Micrographs were made with an Olympus BX50 microscope using bright field.

DNA isolation, amplification and sequencing, and phylogenetic and statistical analyses

Total DNA was isolated from basidiomata and thallus fragments following the protocol described by Reynolds & Williams (2004). Basidioma DNA was used to obtain the sequences of the fungal nuclear large ribosomal subunit gene (LSU) and the internal transcribed spacers of the rRNA operon (ITS), using specific primers (see Supplementary Material Table S1, available online). The DNA from thallus fragments was used to obtain algal sequences. The internal transcribed spacers of the rRNA operon (ITS), the chloroplast *psbB* gene encoding for the protein CP-47 of photosystem II, the 23S rDNA encoding the chloroplast large subunit rRNA and the nuclear gene encoding for the L10A protein of the 60S ribosome (*RPL10A*) were sequenced for algal cells using specific primers (Supplementary Material Table S1). Since thallus fragments were directly used for DNA extraction, we designed specific primers based on the sequence of *Coccomyxa subellipsoidea* (SAG 216-13) to avoid amplification of DNA from other inhabiting organisms (Supplementary Material Table S1). Amplification reactions were carried out by PCR performed in a 25 µl reaction volume containing a PuReTaq™ Ready-To-Go™ PCR bead (GE Healthcare, Buckinghamshire, UK). Cycling conditions for all PCRs were: one cycle at 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. These cycles were followed by a final extension at 72 °C for 10 min. Amplified products were electrophoresed in 1.0% agarose gel and stained with SYBR® Safe (Invitrogen, Eugene, OR, USA) for visualization of the bands. PCR products were purified directly using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified amplicons were sequenced using the BigDye™ Terminator Cycle Sequence Ready Reaction Kit II (Applied Biosystems, Foster City, CA, USA), separated by automated multicapillary electrophoresis, and further analyzed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems). Each sample was sequenced in both directions with the same primers as those used for PCR. All the obtained sequences are available in GenBank with the Accession numbers indicated in Table S2 (Supplementary Material, available online).

The phylogenetic relationships of the *L. meridionalis* mycobiont within that genus were inferred on the basis of the alignment of 15 sequences of the fungal LSU rDNA. Sequences from *Arrhenia epichysium* and *A. lobata* were used as out-group. The identity of the chlorobiont of *L. meridionalis* was determined from the sequences of the four molecular markers: the chloroplast *psbB* and 23S rDNA, and the nuclear ITS and *RPL10A*. The algal sequences were arranged into multiple sequence alignments (MSA) using MAFFT version 7 (Katoh & Standley 2013) on the Guidance web server, available at http://guidance.tau.ac.il (Penn *et al.* 2010*a*, *b*). The GUIDANCE2 algorithm was used to create

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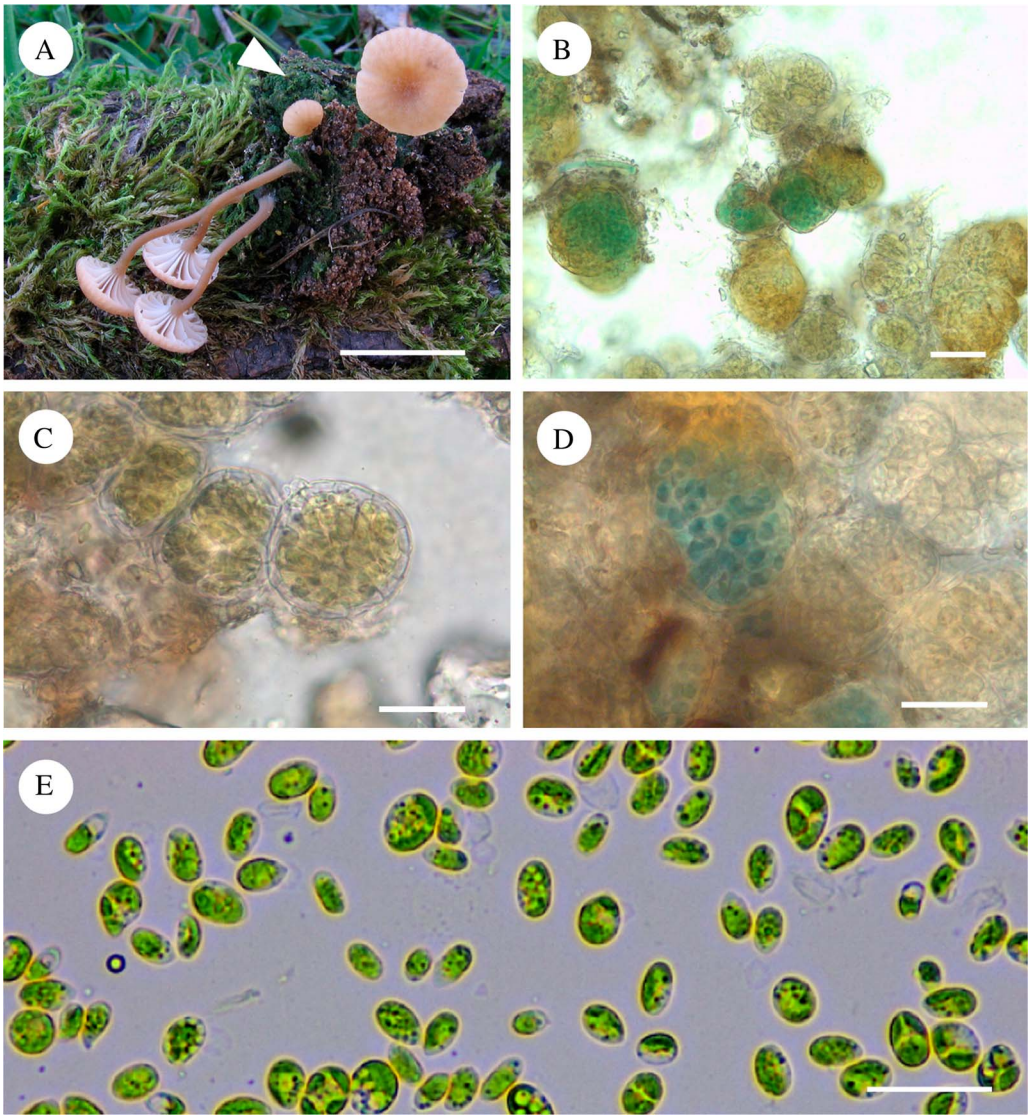

Fig. 1. *Lichenomphalia meridionalis*. A, basidiomata with the vegetative thallus at the base (arrowhead) (AH 32757, Lms 4); B, globules of vegetative thallus (AH 32760, Lms 11); C, vegetative thallus showing globules enclosing *Coccomyxa* algal cells surrounded by mycobiont hyphae (AH 32760, Lms 11); D, vegetative thallus showing globules enclosing a cyanobacterium, which appear as green-blue spots among the *Coccomyxa* cells inside a colourless envelope formed by fungal hyphae (AH 23937, Lms 15); E, chlorobionts isolated from *L. meridionalis* (AH 39243, Lms 12). Scales: A = 5 mm; B–D = 25 µm; E = 20 µm. In colour online.

a SuperMSA by concatenating the default MSA and alternative 20 MSAs. All datasets were subjected to maximum likelihood (ML) with PhyML (Guindon *et al.* 2010) using the general time-reversible model (GTR) as a substitution model (Tavaré 1986), selected as the best model with 'Smart Model Selection' (SMS) (Lefort *et al.* 2017). Bootstrap supports (Felsenstein 1985) were calculated to estimate the robustness of the clades from 100 replicates of the data. Trees were displayed with FigTree v.1.3 (Rambaut 2008). Parsimony networks of haplotypes were constructed according to a 95% parsimony interval with the program TCS 1.18 (Clement *et al.* 2000).

Results

Structure and symbiont composition of Lichenomphalia meridionalis *vegetative thallus*

The vegetative thalli of the *Lichenomphalia meridionalis* samples were of the *Botrydina*-type (Fig. 1B), consisting of green to dark green globules measuring *c.* 20–55 µm diam. with an outer covering of fungal hyphae (Fig. 1B–D). The vegetative thallus of samples Lms1, Lms2, Lms3, Lms6 and Lms 8 consisted exclusively of green globules of 20–55 µm diam. containing green algae. However, the vegetative thallus of samples Lms 4, Lms 5, Lms 7, Lms 9, Lms 10, Lms 11, Lms 12, Lms 13, Lms 14 and Lms 15 contained globules of green algal cells along with globules enclosing a cyanobacterium (see Fig. 1D). The chlorobiont of *L. meridionalis* isolated from a fresh lichen thallus (sample Lms12) was seen as an ellipsoidal, single-celled green alga, measuring 3–5 µm × 6–12 µm (Fig. 1E).

Identification of the L. meridionalis *mycobiont and its phylogenetic relationships within* Lichenomphalia

Fifteen samples of *L. meridionalis* from different localities in Spain (Table 1) were macroscopically characterized by basidioma features. Pilei were ochraceous to yellowish red, convex to applanate

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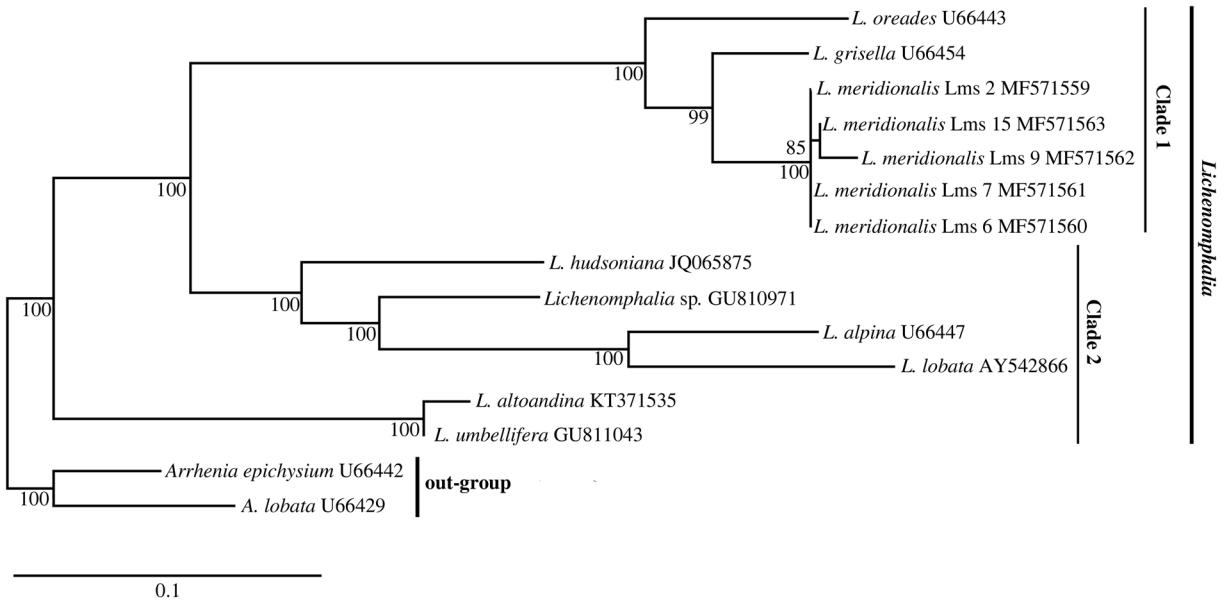

Fig. 2. Phylogram of *Lichenomphalia* species obtained from fungal LSU rDNA sequences. Numbers below branches are ML bootstrapping values. Vertical bars show clades representing subgenera proposed by Lodge *et al.* (2014). The phylogeny was rooted with *Arrhenia* spp. GenBank Accessions are shown to the right of each name. The scale bar corresponds to 0.1 nucleotide substitutions.

or slightly depressed, 4–10 mm in diameter. Stipes 4–10 mm in height had a white mycelial patch at the base and decurrent lamellae concolorous with the pileus (Fig. 1A). We generated five nuclear LSU rDNA sequences of the mycobiont from herbarium specimens, some of them preserved for more than two decades, and these constitute the first molecular data available in the NCBI databases for *L. meridionalis*. These sequences were used to infer the phylogenetic relationships of *L. meridionalis* with other *Lichenomphalia* species. The obtained phylogeny (Fig. 2) showed *L. umbellifera* and the sister species *L. altoandina* occupying the basal position of the fungal LSU phylogram. All the remaining species belonged to a clade with two separate subclades: subclade I included three species, *L. grisella* (accession U66454 originally labelled as *L. velutina*), *L. meridionalis* and *L. oreades* (accession U66443 originally labelled as *L .grisella*), and subclade II included four *Lichenomphalia* species (*L. alpina*, *L. hudsoniana*, *L. lobata* and *Lichenomphalia* sp.). Additional analyses of the nrITS sequences from *Lichenomphalia* species closely related to *L. meridionalis*, such as *L. grisella* and *L. oreades*, revealed the existence of a microindel. This microindel must be a deletion in *L. meridionalis* because the other two taxa form a paraphyletic clade with respect to *L. meridionalis* and thus two independent, but identical insertion events would be needed to explain the observed pattern (Fig. 3A). The phylogenetic reconstruction based on a dataset of fungal nrITS sequences (Fig. 3B) showed the clustering of *L. meridionalis* with *L. grisella* (accession U66454, originally labelled as *L. velutina*), whereas *L. oreades* appeared as sister clade, including two sequences originally labelled as *L. grisella* (U66443, AY293949) but subsequently re-identified as *L. oreades* (Lücking *et al.* 2017*b*).

Identification of Lichenomphalia meridionalis *chlorobionts*

In order to determine the identity of the chlorobiont of *L. meridionalis*, we analyzed several molecular markers. Initially, no ITS sequence could be generated from any herbarium sample. However, a single sequence was obtained from algae isolated from a fresh Lms12 thallus (accession MN473374). BLAST searches carried out against the nucleotide NCBI databases retrieved a best score (470/471 identities) with *Coccomyxa* sp. C8 (accession AY293938) from *Omphalina ericetorum* (currently *L. umbellifera*). This sequence was used to infer the phylogenetic relationships of the chlorobiont from *L. meridionalis* together with other closely related *Coccomyxa* species. The obtained phylogeny (Fig. 4) showed the inclusion of the chlorobiont from Lms12 within a clade designated as *C. subellipsoidea* by Malavasi *et al.* (2016). Since it was impossible to obtain additional ITS sequences from herbarium samples, we decided to use another molecular marker based on the sequence of the chloroplast *psbB* gene of *C. subellipsoidea* (SAG 216-13). The phylogenetic analysis of the chloroplast *psbB* sequences of the chlorobiont from *L. meridionalis* together with other closely related *Coccomyxa* species (Fig. 5) showed a basal lineage represented by *C. dispar* (labelled as strain SAG 49.84), which is the chlorobiont of *Multiclavula vernalis*, and at least four lineages of *Coccomyxa* (L1–L4). Each lineage included algae associated with either Basidiomycota (*Lichenomphalia* and *Multiclavula*) or Ascomycota (*Peltigera* and *Solorina*). Lineage L1 included *C. viridis*, either as chlorobiont of *Peltigera aphthosa* or free-living. Lineage L2 included *C. subellipsoidea* as chlorobiont of *Lichenomphalia* (*L. meridionalis* and *L. umbellifera*). Lineage L3 included *C. simplex* either as chlorobiont of *Solorina saccata* or free-living. Lineage L4 included *C. dispar* as chlorobiont of *Multiclavula vernalis*.

The chlorobionts of *L. meridionalis* were included within L2, which corresponded to *C. subellipsoidea*. The *psbB* sequences of *L. meridionalis* represented three haplotypes ((1) to (3) in Fig. 5A). It is likely that haplotypes (1) and (3) each belong to a different algal strain, L2a and L2b, respectively. Interestingly, sample Lms5 showed double peaks at positions corresponding to differences between haplotypes (1) and (3) (Fig. 5B), which suggests a mixture of both algal strains in this sample. Two previously sequenced samples of *L. umbellifera* had a single haplotype (4). Interestingly, the chlorobiont *C. simplex*, associated with *Peltigerales* such as *Solorina saccata*, was more closely related

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Fig. 3. Alignment and phylogenetic reconstruction based on the fungal nrITS sequences from *Lichenomphalia meridionalis*, *L. grisella* and *L. oreades*. A, alignment of a portion of fungal nrITS2 sequences from each of the three species; GenBank Accession numbers and sample codes are listed adjacent to species names; the discriminator microindel is labelled with asterisks. B, phylogenetic reconstruction based on the fungal nrITS sequences from each of the three species; numbers below branches are ML bootstrapping values, the phylogeny was rooted with *L. hudsoniana*, and GenBank Accession numbers are shown to the right of each name; the scale bar corresponds to 0.1 nucleotide substitutions. In colour online.

to *C. subellipsoidea* occurring in *Lichenomphalia* than to *C. viridis* associated with other *Peltigerales* such as *Peltigera aphthosa*.

Phylogenetic reconstructions and parsimony networks of haplotypes based on sequences of the nuclear *RPL10A* gene allowed the discrimination of four different haplotypes ((1) to (4) in Fig. 6A). The chlorobiont of *L. meridionalis* Lms 4 had nucleotide substitutions found in both haplotypes (1) and (3), as evidenced by double peaks at the corresponding positions (Fig. 6B). Phylogenetic reconstructions and parsimony networks of haplotypes based on sequences of the chloroplast 23S rDNA revealed the presence of three different haplotypes ((1) to (3) in Fig. 6C). Haplotype (1) was found in chlorobionts of *L. umbellifera* and *L. meridionalis* whereas haplotype (2) was found only in chlorobionts of *L. meridionalis*.

The results obtained from sequences for *psbB* (Fig. 5), *RPL10A* (Fig. 6A) and 23S rDNA (Fig. 6B) are consistent with the notion of the existence of two different strains of alga: L2a (in samples Lms 7, Lms 9, Lms 10, Lms 11, Lms 13, Lms 14 and Lms 15) and L2b (in samples Lms 1, Lms 2, Lms 3, Lms 6 and Lms 8), which may coexist in some samples (Lms 4 and Lms 5). It should be noted that the sequences obtained from the isolated chlorobiont (Fig. 1E) were identical to those obtained from the thallus and corresponded to the L2a algal strain. It is noteworthy that samples with the L2b alga were generally collected in localities at higher altitudes than samples with the L2a alga, whereas the samples found to contain both algal strains were generally collected at intermediate altitudes (*c.* 1393 m above sea level (a.s.l.)). Statistical analyses supported the notion that samples containing the L2a alga thrived in localities with an average altitude significantly lower ($P \leq 0.001$) than those with the L2b alga: 1646 versus 1046 m a.s.l., respectively (Fig. 7). Similarly, we found a positive correlation between the association with the L2a strain of chlorobiont and the presence of a cyanobacterium, since all samples associated with L2a chlorobionts carried cyanobacteria. These results suggest the possible existence of an altitude pattern in the appearance of each algal strain associated with *L. meridionalis* and/or the presence of cyanobacteria (Fig. 7). However, it should be noted that the specimens were not collected along one continuous altitudinal gradient, but from various places and at different times.

Discussion

Basidiolichens have been overlooked for a long time because of the very small number of species, which are restricted to special niches. *Lichenomphalia* species are frequently found in arctic and alpine habitats (Oberwinkler 2012), giving rise to the traditional concept of an arctic-alpine distribution. Therefore, the

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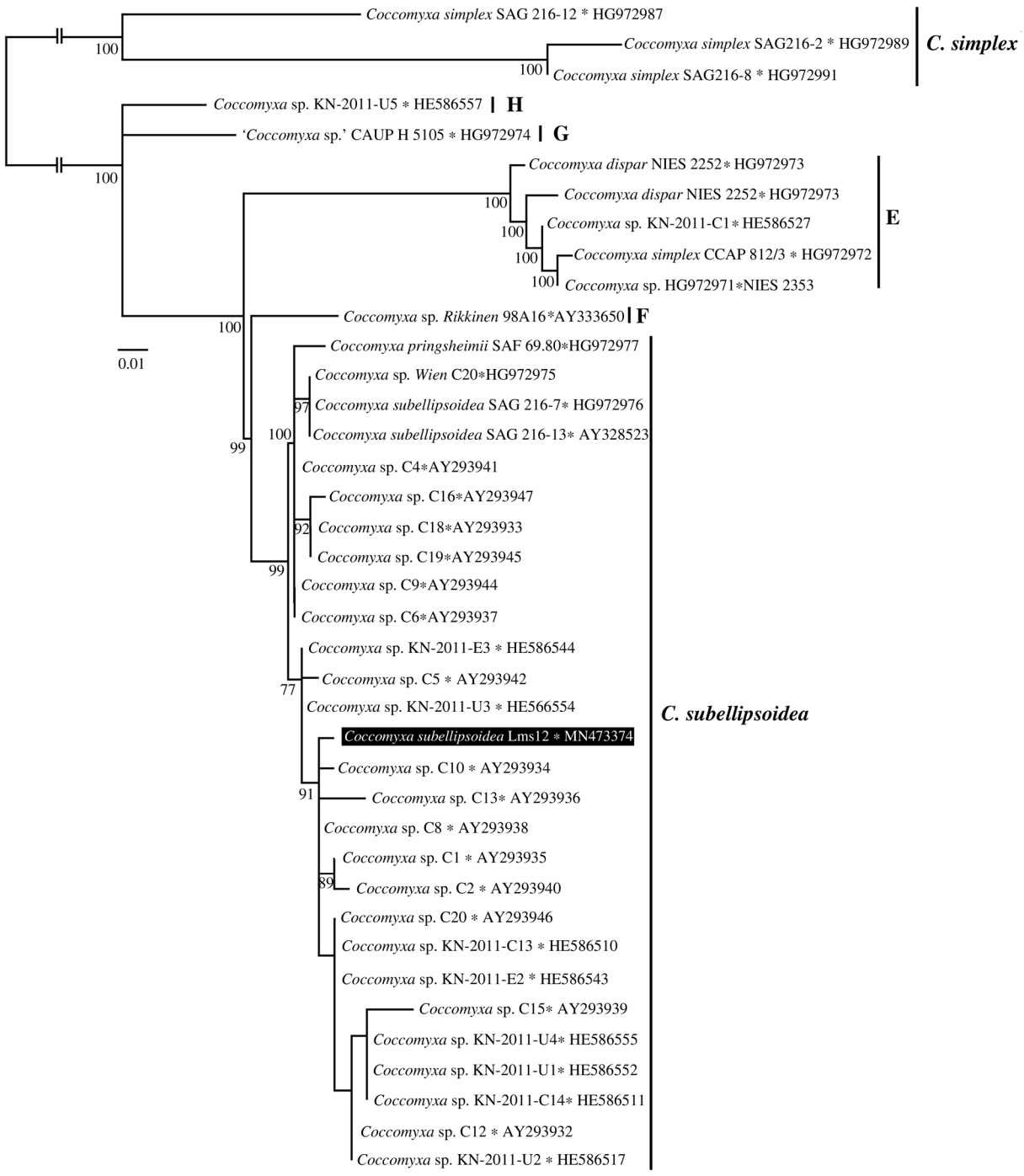

Fig. 4. Phylogram based on the nrITS sequences of the chlorobiont isolated from *Lichenomphalia meridionalis* (Lms12), together with those from other *Coccomyxa* microalgae. Taxon sampling and clade names are according to Malavasi *et al*. (2016). GenBank Accession numbers of the sequences are indicated after the asterisks. Numbers below branches are ML bootstrapping values. The scale bar corresponds to 0.01 nucleotide substitutions. The phylogeny was rooted with *Coccomyxa simplex*.

particular ecology of these species has discouraged research on *Lichenomphalia* in Mediterranean areas such as the Iberian Peninsula. *Lichenomphalia meridionalis* has been frequently mistaken for *L. umbellifera* and the analyzed samples of *L. meridionalis* in the current study showed distinct morphological characteristics of the basidiomata, contrasting with previous reports on those of *L. umbellifera* (Barrasa & Esteve-Raventós 2000; Barrasa & Rico 2001; Barrasa *et al.* 2009). Distinguishing between *Lichenomphalia* species based on morphology is time-consuming and requires considerable expertise on the part of the observer. In our study, we found a microindel within the fungal nrITS2 (Fig. 3A) which can assist in the discrimination of *L. meridionalis* from other similar species. The presence of this indel is consistent with the phylogenetic relationships of *Lichenomphalia* species shown previously (Lücking *et al.* 2017*b*) and in the present study (Fig. 3B). LSU sequences (Fig. 2) show

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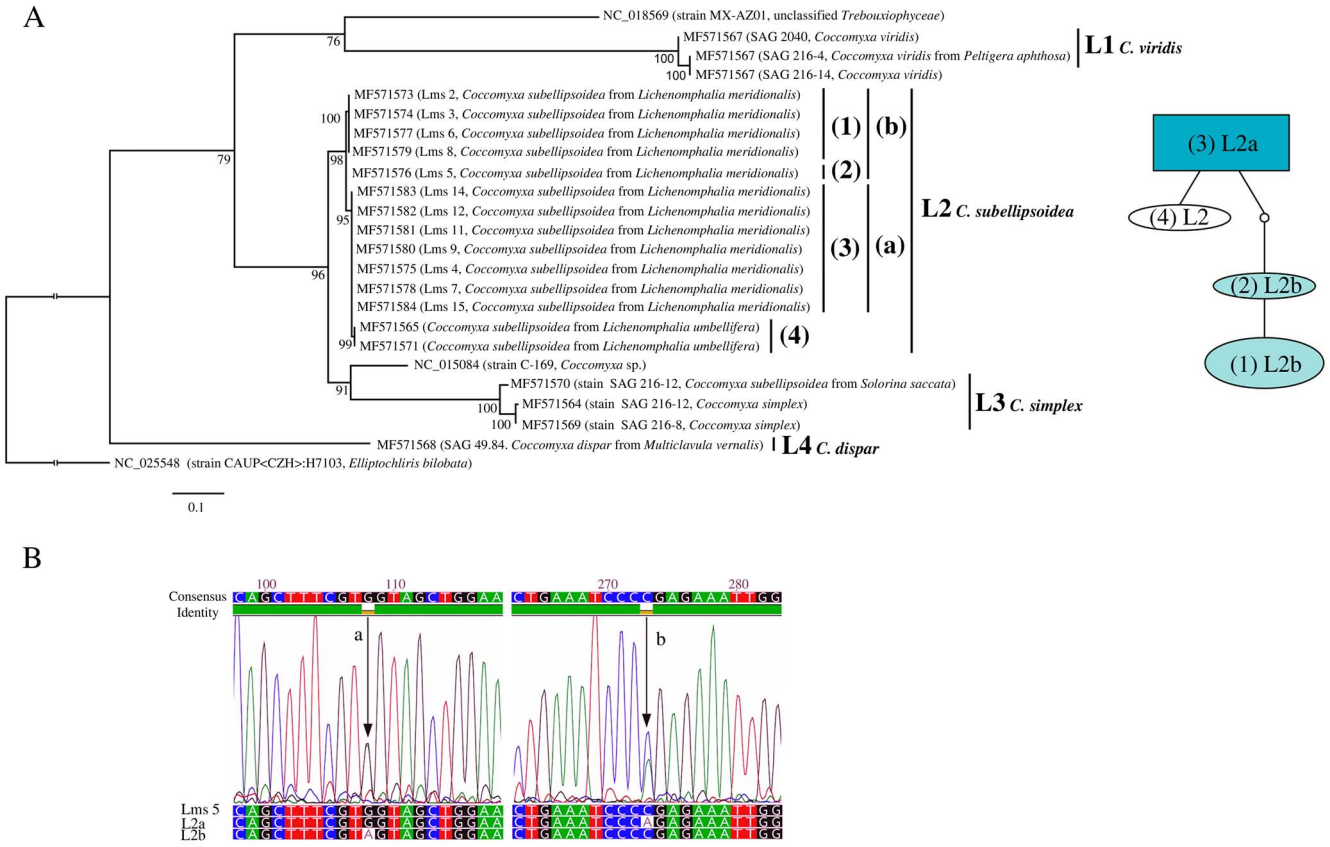

Fig. 5. Phylogram and electropherogram obtained from chloroplast *psbB* sequences of *Lichenomphalia meridionalis* chlorobionts. A, phylogram and parsimony network of haplotypes based on the chloroplast *psbB* sequences of *L. meridionalis* chlorobionts are shown on the left and right, respectively. In the phylogram, numbers above or below branches are ML bootstrapping values. The phylogeny was rooted with *Elliptochloris bilobata* strain CAUP < CZH>:H7103. GenBank Accession numbers are given, and algal species and fungal hosts are indicated in parentheses. Vertical bars delimit each *Coccomyxa* lineage according to Darienko *et al.* (2015) (L1/*C. viridis*, L2/*C. subellipsoidea*, L3/*C. simplex* and L4/*C. dispar*). Sub-lineages or strains are indicated with (a) and (b). Haplotypes found in *L. meridionalis* chlorobionts are indicated as 1 to 4. The scale bar corresponds to 0.1 nucleotide substitutions. In the parsimony network, each rectangle/ellipse corresponds to a particular algal type (1, 2, 3 and 4). Each bar in the network represents a single mutation step and each small empty circle represents an additional change. B, sequence electropherogram of the chloroplast *psbB* gene showing double peaks at specific positions ('a' and 'b', arrowed), which correspond to the differences between the sequences of the *psbB* haplotypes (1), (2) and (3). In colour online.

that phylogenetic relationships between *L. meridionalis* and other related species such as *L. grisella* are comparable to those between other closely related species, such as *L. altoandina* and *L. umbellifera* (which are distinguished according to morphological and molecular data (Sandoval-Leiva *et al.* 2017)). These observations indicate that *L. meridionalis* can also be distinguished from the other *Lichenomphalia* species using molecular tools.

The genus *Lichenomphalia* has *Coccomyxa* species as chlorobionts, as do other basidiolichen genera (e.g. *Multiclavula*) (Nelsen *et al.* 2007). At least five *Lichenomphalia* species (*L. grisella*, *L. hudsoniana*, *L. luteovitellina*, *L. velutina* and *L. umbellifera*) share a single phylogenetic species, *C. subellipsoidea* (Zoller & Lutzoni 2003). The phylogeny of *Coccomyxa* (Fig. 5) is based on the species delimitation described by Darienko *et al.* (2015) and Malavasi *et al.* (2016). In our study, lineages L1, L2 and L4 corresponded to *C. viridis*, *C. subellipsoidea* and *C. dispar*, respectively. Lineage L3 corresponded to *C. simplex* according to Darienko *et al.* (2015) or to *C. simplex* (*C. chodatii* and *C. rayssiae*) and *C. solorinae* (*C. solorinae-saccatae*) according to Malavasi *et al.* (2016). The chlorobionts of *L. meridionalis* occurred within the L2 lineage, which corresponds to *C. subellipsoidea*, and is closely related to the chlorobionts of other *Lichenomphalia* species (see Zoller & Lutzoni 2003). The presence of two different algal strains in the L2 lineage, which appeared to coexist in some samples, indicates that *L. meridionalis* thalli apparently represent a specific and selective form of symbiotic association. Strict preservation of this pattern of algal coexistence probably facilitates the proliferation of this lichen in a very restrictive habitat. In this study, lineage L2a represents *C. subellipsoidea* based on the inferred position of the ITS sequence from Lms12 in the phylogram of Fig. 4 and in relation to SAG 216 strains in Figs 4 and 5. However, the identity of lineage L2b is not clear because of the lack of ITS sequences from any herbarium sample. The phylogram in Fig. 4 is very similar to that of Malavasi *et al.* (2016), which emphasized the importance of ecological differences among *Coccomyxa* species and recognized *C. subellipsoidea* and closely related clades as distinct species units. If L2b represents any such clade (e.g. clade F), then the ecological distinction between L2a and L2b would support the species delimitation criteria proposed by Malavasi *et al.* (2016).

Our finding of globules containing a cyanobacterium in some *L. meridionalis* thalli corresponds to the notion that some other lichens contain both a green microalga (chlorobiont) and a cyanobacterium, which is generally localized in special structures, the cephalodia (Honegger 1993, 2012). *Peltigera aphthosa* (*Peltigeraceae*) is one of the most studied lichens with cephalodia,

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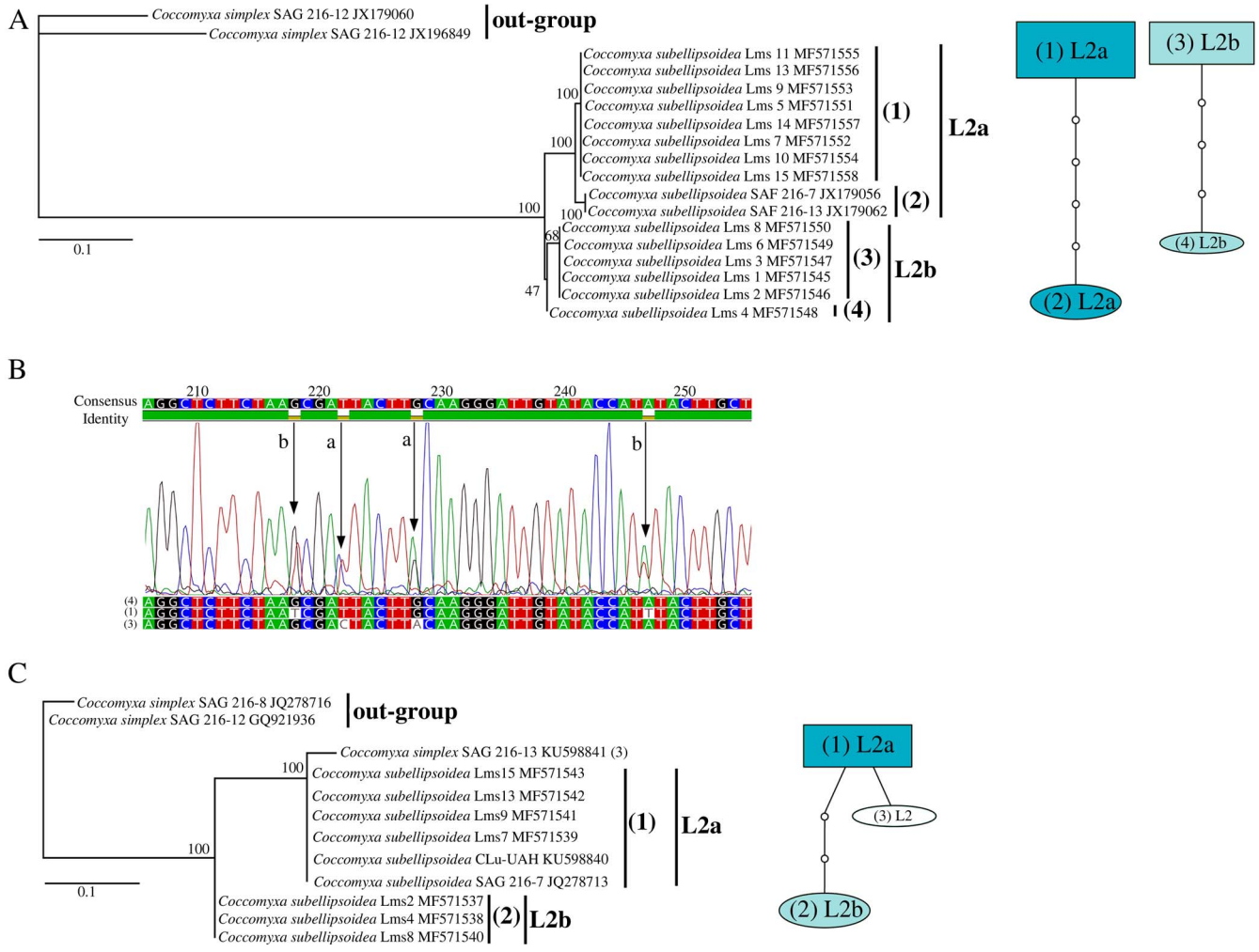

Fig. 6. Phylograms based on the nuclear *RPL10A* and the chloroplast 23S rDNA sequences of *Lichenomphalia meridionalis* chlorobionts and electropherogram of the nuclear *RPL10A* gene. A, phylogram and parsimony networks of haplotypes based on nuclear *RPL10A* sequences of *L. meridionalis* chlorobionts are shown on the left and right, respectively. In the phylogram, numbers above branches are ML bootstrapping values. The phylogeny was rooted with *Coccomyxa simplex*. GenBank Accessions are shown to the right of each name. Vertical bars separate each algal sub-lineage associated with *L. meridionalis* (L2a and L2b). Haplotypes found in chlorobionts are indicated as 1 to 4. The scale bar corresponds to 0.1 nucleotide substitutions. In the parsimony networks, each rectangle/ellipse corresponds to a particular algal type (1, 2, 3 and 4). Each bar in the network represents a single mutation step and each small empty circle represents an additional change. B, sequence electropherogram of the nuclear *RPL10A* gene showing double peaks at specific positions ('a'–'d', arrowed), which correspond to the differences between the sequences of the *RPL10A* haplotypes (1), (3) and (4). C, a phylogram and a parsimony network of haplotypes based on chloroplast 23S rDNA sequences of *L. meridionalis* chlorobionts are shown on the left and right, respectively (for further explanation see A above). In colour online.

containing a green microalga from the genus *Coccomyxa* and a nitrogen-fixing cyanobacterium from the genus *Nostoc* (Büdel & Scheidegger 2008). In this lichen, an increase in the abundance of cephalodia is indicative of higher rates of nitrogen fixation, as a consequence of a lower availability of nitrogen in the environment and serves as a potential marker of nitrogen and phosphorous content in the soil (Asplund & Wardle 2015). Further analyses employing more specific molecular and microscopy techniques will be necessary to demonstrate whether cyanobacteria are stable partners of the *Lichenomphalia* holobiont, or have a commensalist relationship, or whether their coexistence is just due to common ecological preferences. If they indeed represent cephalodia, they can best be compared to internal cephalodia, for instance found in *Lobaria pulmonaria*.

The present study suggests that altitude influences the distribution of the coexisting chlorobionts (strains a and b) associated with the vegetative thallus of *L. meridionalis*. The occurrence of different photobionts along an altitudinal (climatic) gradient has been reported for epilithic lichens (Kroken & Taylor 2000; Blaha *et al.* 2006). Similarly, Řeháková *et al.* (2011) demonstrated that soil phototrophs form complex communities with specific environmental associations and depend on site, altitude and vegetation type. Our results suggest that temperature might limit or facilitate the growth of the two microalgal strains. However, additional environmental factors such as humidity and soil characteristics (texture, pH, availability of mineral nutrients and organic matter content), which do not necessarily depend on altitude, cannot be excluded. Previous studies of the community composition of *Trebouxia* chlorobionts along an altitudinal gradient in the Mediterranean region revealed that both altitude and host genetic identity were strong predictors of photobiont community assembly (Dal Grande *et al.* 2018). The lichen *Ramalina farinacea* establishes associations with two different *Trebouxia* species (TR1 and TR9) that always coexist within the thallus, but their relative proportions vary depending on ecological conditions (Casano *et al.* 2011). *In vitro* experiments have demonstrated that the

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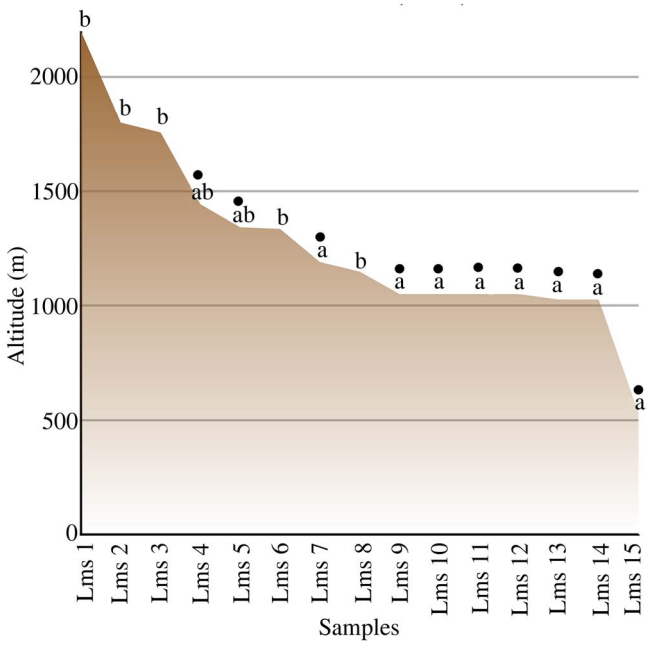

Fig. 7. The relationship between altitude (m above sea level) and occurrence of *Coccomyxa* photobiont strains L2a and L2b in *Lichenomphalia meridionalis*, together with the presence of detectable cyanobacteria associated with the thalli (filled circles). In colour online.

abundance of each species is related to their physiological performance: whereas TR9 grows better under relatively high temperatures and irradiances, TR1 thrives under more temperate and shady conditions. Further studies on the physiological performance of each *Coccomyxa* strain in *L. meridionalis* under different environmental conditions could shed light on the influence of specific environmental conditions on the photobiont composition of this basidiolichen.

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