A case study on the re-establishment of the cyanolichen symbiosis: where do the compatible photobionts come from?

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ABSTRACT

Background and Aims In order to re-establish lichen symbiosis, fungal spores must first germinate and then associate with a compatible photobiont. To detect possible establishment limitations in a sexually reproducing cyanolichen species, we studied ascospore germination, photobiont growth and photobiont association patterns in *Pectenia plumbea*.

Methods Germination tests were made with ascospores from 500 apothecia under different treatments, and photobiont growth was analysed in 192 isolates obtained from 24 thalli. We determined the genotype identity [tRNALeu (UAA) intron] of the *Nostoc* cyanobionts from 30 *P. plumbea* thalli from one population. We also sequenced cyanobionts of 41 specimens of other cyanolichen species and 58 *Nostoc* free-living colonies cultured from the bark substrate.

Key Results Not a single fungal ascospore germinated and none of the photobiont isolates produced motile hormogonia. Genetic analyses revealed that *P. plumbea* shares *Nostoc* genotypes with two other cyanolichen species of the same habitat, but these photobionts were hardly present in the bark substrate.

Conclusions Due to the inability of both symbionts to thrive independently, the establishment of *P. plumbea* seems to depend on *Dendriscocaulon umhausense*, the only cyanolichen species in the same habitat that reproduces asexually and acts as a source of appropriate cyanobionts. This provides support to the hypothesis about facilitation among lichens.

INTRODUCTION

Even though several fungi and/or bacteria are often involved in lichen symbioses, most lichens are predominantly composed of one primary fungal partner (mycobiont) and one or more photosynthetic partners (photobionts), either cyanobacteria or green algae (U'Ren *et al.*, 2012; Werth *et al.*, 2013; Aschenbrenner *et al.*, 2016; Spribille *et al.*, 2016). In sexually reproducing lichens, the association between different symbionts must be re-established during each reproductive cycle. Thus, germinating fungal spores must associate with compatible photobionts in order to initiate thallus morphogenesis (Meeßen and Ott, 2013; Sanders, 2014). This is not the case in many asexually reproducing lichens, which can effectively disperse all partners of the symbiosis into new habitats within their symbiotic propagules (Wornik and Grube, 2010; Sanders, 2014).

In some cyanolichen species, the mycobiont seems to associate only with one specific cyanobacterial genotype. In most cases, the mycobionts are more promiscuous but, still, in general, they rely on a restricted pool of closely related photobionts, some of which may also be shared by other mycobiont species. Lichen photobionts tend to be much less selective, with individual photobiont genotypes commonly associating with several fungal taxa (for a detailed review, see Rikkinen, 2013).

The strategies of re-establishing symbiosis in lichens are diverse. Rikkinen *et al.* (2002) proposed that cyanolichen mycobionts sharing identical photobionts would commonly obtain compatible photobionts from each other, forming photobiont-mediated guilds. Within these ecological communities (i.e. guilds), all fungal species are horizontally linked by their shared photobiont specificity, but may have contrasting roles as sources/recipients of photobionts depending on their prevailing reproductive strategy (Rikkinen, 2003; Belinchón *et al.*, 2015; Cornejo and Scheidegger, 2016). Some lichens can function as 'core species' by producing large quantities of symbiotic propagules. Photobionts from their disintegrating propagules are potentially available to 'fringe species', which only produce fungal spores and do not disperse photobionts themselves. Through such interactions, core species can effectively facilitate the occurrence of fringe species (Belinchón *et al.*, 2015), especially in cases where the photobionts are unable to establish free-living populations. Moreover, while a wide diversity of free-living cyanobacteria occur in terrestrial habitats, most free-living forms do not establish symbiotic

associations with lichen-forming fungi (Rikkinen *et al.*, 2002; Hedenås *et al.*, 2007; Rikkinen and Virtanen, 2008; Rikkinen, 2013, 2017).

Pectenia plumbea (Lightf.) P.M. Jørg. *et al.* is an oceanic cyanolichen species with a continuous but scattered distribution in Europe. Its range extends to the Mediterranean, Atlantic and to the boreal and continental biogeographic regions of Europe (Otálora *et al.*, 2017). On the Atlantic coast, the species is more frequent, although it has lately declined dramatically due to human activity. Although its southern range margin still has viable populations, they are very distanced from each other (COSEWIC, 2010). In central Spain, the species has been listed as vulnerable due to loss and fragmentation of habitat during the past decades (Martínez *et al.*, 2003). Most Spanish populations are small (Martínez *et al.*, 2014) and exhibit low genetic diversity (Otálora *et al.*, 2013), with no detected presence in many seemingly suitable forest habitats prospected in recent field surveys (Martínez *et al.*, 2014). The reasons for its absence are not well understood since the lichen produces small ascospores which could disperse easily – and the mycobiont is not believed to be extremely restricted in its cyanobiont choice (Otálora *et al.*, 2013).

Based on the high reciprocal genetic differentiation and the low genetic diversity of *P. plumbea* found in central Spain, Otálora *et al.* (2013) suggested a limited long-distance dispersion of this lichen. However, in a recent study, Cardós *et al.* (2017) emphasized the apparent lack of dispersal limitations of this species, but suggested that establishment limitations might play a decisive role. The possible difficulties in the establishment might be in the ascospore germination and/or the finding of a compatible photobiont. In this respect, under laboratory conditions, it has been demonstrated that ascospores of most cyanolichens do not readily germinate (Crittenden *et al.*, 1995; McDonald *et al.*, 2013), and may require the presence of compatible photobionts to induce germination and further growth (Meeßen and Ott, 2013).

The cyanobionts of *P. plumbea* belong to a monophyletic lineage of *Nostoc* found in all cyanolichens of the so-called *Nephroma* guild (Rikkinen *et al.*, 2002; Myllys *et al.*, 2007; Kaasalainen *et al.*, 2015). These cyanobionts have not been observed as free-living populations (Rikkinen, 2013). Attempts to bring them into pure culture have been unsuccessful (Jordan and Rickson, 1971; Kardish *et al.*, 1989), and the cyanobionts do not seem to produce hormogonia,

i.e. short, small-celled filaments that move by gliding, and play an essential role in the establishment of many cyanobacterial symbioses (Adams *et al.*, 2013; Rikkinen, 2017).

In order to identify which factors are hindering the establishment of this threatened lichen species in the Spanish forests, we studied both symbionts from different perspectives: cyanobiont diversity (at the species and community level) and lichen life cycle (including ascospore germination and photobiont recruitment). In particular, we analysed (1) the photobiont diversity of nine *P. plumbea* populations in different zones of Spain; (2) the photobiont diversity of co-inhabiting epiphytic cyanolichen species of the same habitat; (3) the germination of *Pectenia* ascospores; and (4) the diversity and growth of *Nostoc* photobionts isolated from *Pectenia* thalli and from their substrates.

MATERIALS AND METHODS

Study sites

Nine Spanish forest sites with *Pectenia plumbea* populations were studied, four (I–IV) of them in the north-western part, three (V–VII) in the central part and two (VIII–IX) in the south-eastern part of the country (Table 1; Supplementary Data Fig. S1). All studied forests are unmanaged and have a closed canopy of native old-growth trees (Otálora *et al.*, 2013, 2015; Martínez *et al.*, 2014; Cardós *et al.*, 2016).

Diversity and growth of lichen-symbiotic and free-living cyanobacteria

To determine the overall level of cyanobacterial diversity in *P. plumbea* in the Iberian Peninsula, we first sampled 3–4 *Pectenia* thalli collected from each of the nine forest sites (Table 1). Subsequently, we sampled intensively in one 15 × 15 m study plot established at Gargantilla (forest site V) in Central Spain (Supplementary Data Fig. S1). This studied plot had 21 standing trees, 17 of which had *P. plumbea* and/or other epiphytic cyanolichens growing on the trunk {i.e. *Collema furfuraceum* (Arnold) Du Rietz, *Fuscopannaria mediterranea* (Tav.) P.M. Jørg., *Nephroma laevigatum* Ach. and the cyanobacterial state of *Lobaria amplissima* (Scop.) Forssell [*Dendriscocaulon umhausense* (Auersw.) Degel.] from hereon]}. In each tree, two 10 × 10 cm grids were placed: one at breast height (130 cm) and the second close to the trunk base,

avoiding the lowermost section with terricolous lichens and bryophytes. One specimen of each cyanolichen species present on each grid was collected for DNA analysis, adding up to a total of 71 cyanolichen specimens from 31 grids on the 17 studied trees (Table 2). It was also checked that all the species collected within the grids represented the entire epiphytic cyanolichen diversity present in the study plot and its surroundings, and no other epiphyte cyanolichen species were found.

In order to determine the cyanobacterial diversity of the substrate in which cyanolichens were growing, cyanobacterial cultures were developed from each of the 42 grids placed on the 21 trees present in the plot, mixing exposed bark, epiphytic bryophytes and/or green algal lichens. Cyanobacterial cultures were initiated by touching the sterile surface of the solid growth medium (nitrogen-free Z8) with small pieces of the wild substrates. Cultures from each grid were grown at room temperature and indirect daylight for 68 d. The emerging cyanobacterial colonies were first classified according to their shape, size and colour under the dissecting microscope. Only colonies corresponding to *Nostoc* (with non-branched isopolar trichomes, spherical or cylindrical cells, and heterocysts) were studied further. From these colonies, 17 isolates which did not produce hormogonia but rather formed dark green, pearlshaped colonies ('*Nostoc punctiforme*') were first selected for DNA extraction (*Nephroma* guild cyanobionts do not seem to produce hormogonia in culture). DNA was also extracted from 41 additional colonies representing other *Nostoc*-like colony morphologies.

To determine photobiont growth, we used the complete geographic sampling and collected 24 fresh *P. plumbea* thalli from the nine populations (2–4 specimens per population). In the laboratory, cyanobacterial photobionts were isolated after carefully removing the upper cortex of the dry lichen thalli with a sterile needle. From each thallus, eight minute fragments of the photobiont layer (0.01–0.04 mm2) were placed separately on Petri dishes with nitrogen-free Z8 medium. The isolates were kept at room temperature under indirect daylight, and periodically checked for photobiont growth and/or production of hormogonia during the following 6 months. To document growth, all cyanobacterial isolates from the sites V, VI and VII (16 isolates obtained from two *Pectenia* thalli per site) were photographed on the day of isolation and after 50 d of culture. As control, we repeated the same isolation procedure with the same growth condition for two terricolous cyanolichen species, *Peltigera canina* (L.) Willd.

and *Peltigera praetextata* (Flörke) Vain., collected from localities V, VI and VII (two specimens per species and locality, with 96 isolates in total).

The initial and final size of the photobiont isolates was quantified using the software ImageJ v.1.49 (Rasband, 1997–2015), calculating the relative area growth rate (RAGR) using the formula: RAGR (mm2 cm–2 d–1) = ln (*A*f/*A*i)/Δ*t* × 100, where *A*f is the final area, *A*i the initial area and Δ*t* is the duration of the experiment (Evans, 1972). To determine the statistical significance of differences in the RAGR between cyanobacterial isolates from *P. plumbea* and the *Peltigera* species, we used analysis of variance (ANOVA) including collection site and lichen thallus as explanatory factors.

Germination of fungal ascospores

To determine ascospore germination, we obtained ascospores from two *P. plumbea* populations (VI and VII) in central Spain. These sites have the greatest abundance of the species of all known localities in the Mediterranean region (Martínez *et al.*, 2014). From each population, small pieces including apothecia were collected from 25 lichen thalli. A total of 500 apothecia (ten from each lichen specimen) were cleaned and washed following Yoshimura *et al.* (2002). Seven apothecia were attached per Petri dish lid with petroleum jelly under sterile conditions. The distance from the apothecial surface to the culture medium was 1–2 mm. The Petri dishes were then inverted and stored in the dark at 20 °C, and apothecia discharged ascospores upwards to the medium.

We used four different media for the germination experiments (Supplementary Data Appendix 1): agar, MYA, LBM (Yoshimura *et al.*, 2002) and BBM (Deason and Bold, 1960), prepared with four liquid solutions in all possible combinations (16 culture media in total). The four distinct liquid solutions were prepared as follows. (1) Preparation with bark collected from the same tree species on which the lichen specimen had grown. The extract was prepared following the protocol of Stocker-Wörgötter and Türk (1991), substituting the 500 g of soil with 250 g of bark. (2) Preparation with lichen extract made by crushing and boiling *P. plumbea* thallus fragments. The concentration of *Nostoc* cells in the extract was adjusted to 0.2 g L–1, which was previously found to promote ascospore germination in some cyanolichens (Scott, 1964). (3) Preparation with the absorbent cyclodextrin (10 g L–1), an absorbent that reduces germination autoinhibition, a phenomenon which was previously found to induce ascospore

germination in some cyanolichens (Denison, 2003). (4) Preparation with distilled water as control.

The amount of discharged ascospores was recorded every 24 h. After 72 h, ascospore discharges ceased and the lids with apothecia were replaced with sterile lids. All ascospore cultures were kept in sterile conditions at 18–21 °C and inspected visually every 72 h for 60 d for any evidence of spore germination. The possible effect of light on germination was investigated by exposing the cultures to two contrasting light regimes: half of the plates were kept in total darkness and the other half exposed to a normal daylight cycle (16 h photoperiod, 100 µmol photons m–2 s–1). Spore discharge occurred in all but two treatments (agar/bark extract/light and LBM/lichen extract/light), so that a total of 30 treatments could be tested. As a germination control, we placed four *Xanthoria parietina* (L.) Th. Fr. (a green-algal lichen) apothecia on four different Petri dishes with BBM and exposed them to the same growth conditions as the *Pectenia* samples. *Xanthoria parietina* was selected as a control because it performs very well in culture under a range of conditions and culture media (Molina and Crespo, 2000).

Molecular data

We used the cyanobacterial tRNALeu (UAA) intron for determining the genetic diversity of cyanobacteria in lichen thalli and cyanobacterial cultures (Paulsrud and Lindblad, 1998; Costa *et al.*, 2002; Olsson *et al.*, 2012; Kaasalainen *et al.*, 2015).

DNA from cyanolichen specimens was extracted using a GeneJET™ genomic purification kit (Fermentas, Helsinki, Finland). The *trnL* genes from lichen specimens were amplified using the primers tRNALeu outF and outR (Paulsrud and Lindblad, 1998). PCR amplifications were performed in a 50 µL volume, containing 4 µL of diluted genomic DNA, 1 µL of each primer (50 µm), 5 µL of 10× buffer, 1 µL of 10 mm dNTPs (Fermentas), 1.25 µL of 20 mg mL–1 bovine serum albumin (BSA; Fermentas), 1.25 µL of 2 U µL–1 Dynazyme II (Fermentas) and 35.5 µL of water to complete the total volume. Amplifications were performed as follows: initial denaturation at 94 °C for 3 min, followed by four cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, followed by 26 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The PCR products were purified using a GeneJet™ PCR Purification Kit (Fermentas). For the cyanobacterial cultures, DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA); PCR amplifications were performed using a Multiplex PCR Kit (Qiagen) in a

25 µL volume, containing 3 µL of diluted genomic DNA, 1 µL of each primer (tRNALeu outF and outR, 10 µm) and 20 µL of distilled water. Amplifications were performed using the program described in Schultz *et al.* (2015). The PCR products were purified using Exo-sap-IT (USB Corporation, Santa Clara, CA, USA).

The purified PCR products were sequenced by Macrogen Inc. using the same amplification primers. After sequencing, the chromatograms of all sequences were checked using EbioX (version 1.5.1) and aligned with MAFFT (version 7).

All obtained sequences are listed in Supplementary Data Table S1. Sequences are deposited in GenBank (accession numbers: LT839425–LT839602).

RESULTS

Cyanobacterial diversity of cyanolichens

A total of six different cyanobacterial tRNALeu (UAA) intron sequences (genotypes A1, A2, A3, A5, A6 and B1) were obtained (Tables 2 and 3) from 101 epiphytic cyanolichen thalli collected from the nine study sites. The length of the sequences ranged from 350 to 380 bp, all belonging to *Nostoc*, as confirmed by blast searchers.

The sequences obtained from all 60 *Pectenia plumbea* specimens from the nine forest sites belonged to only five closely related *Nostoc* genotypes (Tables 2 and 3), which all had a *Nephroma*-type Class I repeat motif in the P6b region (Fig. 1) (Costa *et al.*, 2002). Two of them (A1 and A3) were previously known from other *Nephroma* guild cyanolichens (Kaasalainen *et al.*, 2015). Cyanobacterial sequences obtained from the 71 cyanolichen specimens sampled in Gargantilla belonged to four *Nostoc* genotypes (Tables 2 and 4). At this site, *Pectenia* cyanobionts belonged to only three different *Nostoc* genotypes (A–A3), two of which (A1 and A2) were also found in the complete sampling across the Iberian Peninsula (Table 3). An identical spectrum of *Nostoc* genotypes (A1–A3) was detected in *Nephroma laevigatum* specimens from Gargantilla. One of these genotypes (A2) was found in all *Dendriscocaulon umhausense* specimens, and another genotype (A3) in all *Fuscopannaria mediterranea* specimens analysed (Table 2). The fourth *Nostoc* genotype (B1) found in Gargantilla was exclusively present in the six *Collema furfuraceum* specimens from this site. The sequence of

this fourth genotype differed significantly from the three others in having a Class II repeat motif in its P6b region (Tables 2 and 4).

Diversity of cultured cyanobacteria

A total of 58 cyanobacterial tRNALeu (UAA) intron sequences were obtained from cyanobacteria cultured from substrate samples (Table 4; Supplementary Data Table S1). Only three sequences (one isolate with *Nostoc* genotype A2 and two with A3) were identical to those of *Nostoc* genotypes from *Pectenia* and other *Nephroma* guild cyanolichens. Six cultured isolates were identical to the *Nostoc* cyanobiont of *C. furfuraceum*; this genotype (B1) had been amplified previously from some other *Collema* guild cyanolichen species (Kaasalainen *et al.*, 2015). The remaining 49 isolates represented 22 different genotypes of *Nostoc* or other nostocalean cyanobacteria (Table 4). Most of them had never been sequenced before, and have not been reported from epiphytic cyanolichens (Supplementary Data Table S1), the sole exception being one isolate of *Nephroma*type genotype A4 (Fig. 1). These genotypes (except A4) had either Class II or *Collema*-type Class I repeat motifs in the P6b region and were thus easily distinguished from the monophyletic group of *Nostoc* genotypes that characterize all *Nephroma* guild cyanolichens (Kaasalainen *et al.*, 2015).

Ascospore discharge and germination

In *P. plumbea*, 34.8 % of all apothecia discharged ascospores into the culture medium. The mean production of spores per apothecium was 29.2 (s.d. ± 30.1, min = 1, max = 135). During 60 d of culture, none of the spores germinated in any of the 30 different treatments (1461 spores in total). Each of the *Xanthoria parietina* apothecia that were used as control discharged 100–200 spores and >95 % of these spores germinated during the first 7 d of culture.

Photobiont growth and reproduction

All 192 *Nostoc* cultures isolated from *P. plumbea* thalli remained without morphological modifications and apparently alive after the first 50 d of culture, but none of them produced hormogonia (Supplementary Data Fig. S2). Their mean growth rate (RAGR ± s.d.) was 0.7 ± 0.7 mm2 cm–2 d–1. The growth rates of *Nostoc* cyanobionts isolated from *P. canina* and *P. praetextata* (controls) were 4.5 ± 2.6 and 2.8 ± 1.4 mm2 cm–2 d–1, respectively. The observed

difference in the growth of *Nostoc* cyanobionts isolated from *P. plumbea* and the two *Peltigera* species was statistically significant (F = 278.1, P < 0.001, R2 adj = 0.8). The rapid growth of *Peltigera* cyanobionts was attributed to the abundant production of hormogonia (Supplementary Data Fig. S2), which occurred in 57.1 % of their isolates. After 6 months of culture, most *Nostoc* isolates (82 %) from *P. plumbea* remained alive (a few had succumbed to fungal contamination), but none of them had produced hormogonia.

DISCUSSION

The results obtained in this study provide important new insight into two critical phases in the life cycle of lichens. Specifically, the strict constraint found for ascospore germination and the existence of shared photobiont genotypes between different cyanolichen species both suggest that facilitation interactions are crucial in the early establishment of new *Pectenia plumbea* thalli.

After dispersal, the fungal ascospore must first germinate to re-establish the symbiosis with the cyanobacterial partner. We found no evidence of spore germination under any of the 30 culture conditions tested, despite the fact that an abundance of spores was discharged (Crittenden *et al.*, 1995). Inbreeding problems cannot explain this result as the experimental materials were collected from the most abundant (Martínez *et al.*, 2014) and genetically diverse (Otálora *et al.*, 2013) Mediterranean populations of *P. plumbea*. Previous attempts to germinate cyanolichen ascospores have also often been unproductive (Crittenden *et al.*, 1995; McDonald *et al.*, 2013), with poor development of the germ tube, or interruption of growth soon after (Crittenden *et al.*, 1995; Sangvichien *et al.*, 2011). However, in *Peltigera* spp., fungal spore germination can be triggered by adding the photobiont into the culture (Scott, 1964), and in some cases even resynthesis has been successful with this technique (Ahmadjian, 1989; Stocker-Wörgötter and Türk, 1991). Conversely, as we have shown here, the very slow growth observed in the photobiont of *P. plumbea* makes it difficult to culture and it may also hinder fungal spore germination under laboratory conditions, as found previously for other *Nephroma* guild cyanolichens (Kardish *et al.*, 1989; Rikkinen *et al.*, 2002). As it is very unlikely that *P. plumbea* ascospores are always non-viable, it is probable that the spores may require the presence of a

sufficient amount of compatible photobionts in the habitat to initiate germination (Meeßen and Ott, 2013).

Therefore, we isolated and grew *P. plumbea* photobionts not only to test whether addition of the photobionts to ascospore cultures would induce ascospore germination but also to determine whether the photobionts have the ability to survive and spread as free living. The photobionts isolated from *P. plumbea* thalli showed slow but positive growth, and survived in culture for extended periods; however, none of them produced hormogonia. The very slow growth and apparent inability to produce hormogonia may be related to a high level of gene erosion, as suggested for other symbiotic cyanobacteria where a large proportion of pseudogenes indicated that the genome may be in a state of degradation (Ran *et al.*, 2010).

In contrast to *P. plumbea*, the *Peltigera* isolates used here as control grew well and readily produced large amounts of hormogonia. Thus, the evolution of lichen-symbiotic cyanobacteria seems to have resulted in some *Nostoc* genotypes almost completely losing the ability for autonomous growth needed for an aposymbiotic way of life. Failure to complete the typical life cycle of *Nostoc* may prevent such symbiotic genotypes from thriving outside lichen thalli; thus, these genotypes will not represent a quantitatively significant source of photobionts for the establishment of *P. plumbea* ascospores, or indeed the spores of other fungal species associated with the same *Nephroma* guild *Nostoc* genotypes.

This being the case, what is the source of compatible photobionts, at the community level, that enables the establishment of new *Pectenia* thalli? From the huge diversity of free-living colonies obtained from substrate cultures, we selected all the colonies that phenotypically resembled *Nephroma* guild *Nostoc* for genetic identification. Only three out of 58 colonies matched the symbiotic genotypes which are present in lichen thalli. Because lichen establishment is dependent on the density of propagules dispersed (Dettki *et al.*, 2000), the extreme sparsity of appropriate *Nostoc* colonies available for dispersing ascospores cannot contribute to the establishment of a great number of new thalli. In a recent study, Zúñiga *et al.* (2017) analysed the diversity of photobionts present in the substrates of several *Peltigera* species and found an overlap between the phylotypes of the lichen cyanobionts and those present in their substrates, suggesting that the latter were a possible source of lichen photobionts. In our case, the most probable source of the three colonies of symbiotic *Nostoc* found from substrate cultures were microscopic symbiotic propagules of *F. mediterranea* or *D.*

umhausense, or similarly cryptic minute thallus fragments of *P. plumbea*, *N. laevigatum* or of the two previously mentioned species (Rikkinen *et al.*, 2002; Nelsen and Gargas, 2008; Wornik and Grube, 2010). Once such aposymbiotic colonies are established outside lichen thalli, they can potentially remain as 'free-living epiphytes' for some time – partly thanks to the gelatinous sheath surrounding the colonies that confers greater desiccation tolerance and minimizes damage from UV radiation (Potts, 1996).

The other 55 free-living cyanobacterial colonies that morphologically resembled '*Nephroma*-type' *Nostoc* genotypes were not detected in any other local cyanolichen species (Table 4; Supplementary Data Table S1). The high diversity of cyanobacteria in the substrate highlights a major difference in genetic composition between assemblages of symbiotic and free-living *Nostoc* from the same substrate and suggests that for some fungal ascospores the primary source of compatible photobionts must be within the resident lichen community, although for others the substrate can also act as a source of photobionts (Zúñiga *et al.*, 2017).

At the community level, it is remarkable that all four cyanolichens analysed in this study associated with only four different *Nostoc* genotypes even when a plethora of other cyanobacterial genotypes were present in the substrate. Some studies have suggested that mycobiont–photobiont interactions are modulated by climate, with a higher specificity observed in warmer regions (Fernández-Mendoza *et al.*, 2011; Singh *et al.*, 2017). The hot Mediterranean climate of our study area may partly explain the high selectivity of the four cyanolichens investigated here (all four species have a world-wide distribution, albeit with clear oceanic requirements). The local community included four *Nephroma* guild species, which are able to exploit almost all compatible *Nostoc* symbionts that are locally available. Conversely, *C. furfuraceum*, the single local member of the *Collema* guild, associated with only one *Nostoc* genotype in spite of the wide diversity of available *Collema*-type *Nostoc* genotypes, and so it can be considered highly selective (Beck *et al.*, 2002). This result underscores the importance of the guild structure when analysing mycobiont–cyanobiont association patterns, and gives support to a high selectivity in lichen symbiosis at a community scale (Myllys *et al.*, 2007; Fedrowitz *et al.*, 2011; Chagnon *et al.*, 2018).

Regarding photobiont selectivity of individual species, we found different patterns in relation to species reproductive mode. The two species reproducing sexually (*N. laevigatum* and *P. plumbea*) share the same three *Nostoc* genotypes. The other three symbiotically dispersing

species (*F. mediterranea*, *C. furfuraceum* and *D. umhausense*) showed no evidence of photobiont switch or horizontal transmission, since each of them always harboured their own specific photobiont. A lower selectivity in sexual species might constitute an adaptive trait to enable effective establishment (Wirtz *et al.*, 2003; Fedrowitz *et al.*, 2011; Magain *et al.*, 2018), since these species must re-encounter an appropriate photobiont during each dispersal cycle. The sexual species were most frequently associated with *Nostoc* genotype A2 (87 and 71 % of cases in *N. laevigatum* and *P. plumbea*, respectively), which might reflect its optimal performance in the studied habitat (Werth and Sork, 2014). It is even possible that symbiotic combinations with the less frequent *Nostoc* genotypes (A1 or A3) might constitute a temporal solution until the optimal photobiont can be acquired, as was demonstrated previously (Ott, 1987; Beck *et al.*, 1998). Interestingly, in the studied lichen community, the dominant genotype A2 can only be effectively dispersed and thus facilitated by *D. umhausense*, which is a relatively less abundant species, both regionally (see online appendix 1 in Cardós *et al.*, 2016) and locally (pers. obs.), than *F. mediterranea*, which harboured and effectively disperses the less common genotype A3. Presumably, the number of propagules dispersed with the genotype A3 must be greater than that of A2, but sexual species appear to associate preferentially with A2, even though this genotype is less available. These observations suggest that the role of *D. umhausense* may be crucial for the establishment of the sexual species and hence for the configuration of the cyanolichen community. The influence of *D. umhausense* on *N. laevigatum* is likely to be lower since *N. laevigatum* is able to acquire more cyanobionts from *F. mediterranea* propagules than *P. plumbea* (with only one thallus with genotype A3, Table 2). The greater dependence of *P. plumbea* (a vulnerable species) on the propagules of only one species, which in turn is regionally rare, might explain its threatened status. On the other hand, *D. umhausense*, the cyanobacterial state of *L. amplissima*, is very rare in the north of Spain (Burgaz and Martínez, 2003), being more frequent in Mediterranean areas; this scarcity might drive the spores of *P. plumbea* to acquire *Nostoc* from other cyanolichens. Our results on regional photobiont associations show that *P. plumbea* individuals from northern populations acquire *Nostoc* from various other sources, indicating that there is higher selectivity at the local scale than regionally. Indeed, we found no clear pattern at the regional scale, as demonstrated previously in the *Nephroma* and *Peltigera* genera (Fedrowitz *et al.*, 2012; Magain *et al.*, 2017). Thus, in central Spain, *P. plumbea* may depend exclusively on the occurrence of *D. umhausense*,

which can be considered locally as a pioneer for sexual species to colonize new habitats. Seeking additional evidence to support this hypothesis, we analysed data from previous abundance surveys of 588 plots in the Spanish Mediterranean region (G. Aragón, unpubl. res.) and found that *D. umhausense* occurred in 111 out of the 588 plots. *Pectenia plumbea* occurred only in 26 but, crucially, in 25 out of these 26 plots *D. umhausense* was also present (Supplementary Data Table S2). This finding demonstrates that the occurrence of *P. plumbea* is dependent on the presence of the asexually reproducing *D. umhausense*.

CONCLUSIONS

Our results provide important new support for the photobiont-mediated lichen guild hypothesis. In our case study, the establishment of the two 'fringe species' is quantitatively dependent on the propagules produced by a unique 'core species', that disperses its photobiont (*Nostoc*) strain effectively via its symbiotic propagules (isidia). This constitutes a clear case of lichen facilitation. The 'core species' also benefits from the situation as a proportion of its photobionts that are dispersed onto suboptimal substrates are 'scavenged' into the thalli of other guild members. Our results also show that tree bark hosts a great cryptic cyanobacterial diversity, therefore studies on lichen photobiont diversity (Hedenås *et al.*, 2007) and lichen facilitation (Svensson *et al.*, 2016) should seek to identify genetically the colonies in order to quantify the true extent of compatible strains present in the substrate.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic. oup.com/aob and consist of the following. Appendix 1: detailed method description. Figure S1: location of the studied populations and image of *Pectenia plumbea* and its habitat. Figure S2: example of the growth of cyanobacterial photobionts isolated from lichen thalli. Table S1: nucleotide sequences of the genotypes identified in the study. Table S2: data from previous abundance surveys showing the

coexistence of *Pectenia plumbea*, *Dendriscocaulon umhausense* and *Fuscopannaria mediterranea*.

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TABLES

Table 1. Collecting localities and the experiments for which each *Pectenia plumbea* population was used. Experiments: 1, overall level of cyanobacterial diversity in Pectenia plumbea; 2, free-living and symbiotic cyanobacterial diversity in a cyanolichen community; 3, quantification of photobiont g. Co-ordinates correspond to the ETRS89 system.

Population number	Locality name and Province	Host tree	Co-ordinates N (m)	Co-ordinates W (m)	Experiments
North-west					
I	Muniellos, 'Fuentes del Narcea, Degaña and Ibias Natural Park', Asturias	*Corylus avellana*	4767227	688741	1, 4
II	Moal, 'Fuentes del Narcea, Degaña and Ibias Natural Park', Asturias	*Quercus robur*	4768894	690894	1,4
III	Monasterio de Hermo, 'Fuentes del Narcea, Degaña and Ibias Natural Park', Asturias	*Fraxinus excelsior*	4761881	698987	1, 4
IV	Rebollar, 'Fuentes del Narcea, Degaña and Ibias Natural Park', Asturias	*Fraxinus excelsior* mixed	4768471	692074	1, 4
Centre					
V	Gargantilla, 'Cabañeros National Park', Ciudad Real	*Arbutus unedo*	4367010	363513	1, 2, 3, 4
VI	El Chorro, 'Cabañeros National Park', Ciudad Real	*Quercus ilex* spp. *ballota*	4379334	358474	1, 3, 4, 5
VII	Navas de Estena, 'Cabañeros National Park', Ciudad Real	*Quercus ilex* spp. *ballota*	4373385	367094	1, 3, 4, 5
South-east					
VIII	Sierra Madrona, 'Valle de Alcudia and Sierra Madrona Natural Park', Ciudad Real	*Quercus pyrenaica*	4256065	389760	1, 4
IX	Río Mundo, 'Calares del Mundo and Sima Natural Park', Albacete	*Quercus ilex* spp. *ballota*	4256629	549190	1, 4

Table 2. Genotypes for the tRNALeu (UAA) intron of the symbiotic *Nostoc* from all the lichen species of the community at site V (Gargantilla). Rows represent the place on each tree (base or bole) where the specimen of each species (columns) was sampled. Base: close to the trunk base, but avoiding the lowermost section of the truck. Bole: at breast height (130 cm). Species reproducing sexually are underlined; the others reproduce asexually (the photobiont is dispersed within the propagule). The abbreviated species are: *Nephroma laevigatum*, *Fuscopannaria mediterranea*, *Collema furfuraceum* and *Dendriscocaulon umhausense*. Dashes represent the absence of the species at that sampling unit. □, A1, ▲, A2; ○, A3, + , B1.

		Pectenia plumbea	*N. laevigatum*	*F. mediterranea*	*C. furfuraceum*	*D. umhausense*
Tree 1	Base	▲	○	–	–	–
	Bole	▲	▲	○	–	–
Tree 2	Base	▲	–	–	–	–
	Bole	▲	–	–	–	–
Tree 3	Base	□	▲	○	–	▲
	Bole	▲	–	○	–	–
Tree 4	Base	▲	▲	–	–	–
	Bole	▲	□	–	–	▲
Tree 5	Base	▲	▲	–	–	▲
	Bole	▲	–	–	–	–
Tree 6	Base	▲	▲	–	–	–
	Bole	▲	▲	○	–	–
Tree 7	Base	▲	▲	–	–	–
	Bole	▲	–	○	–	▲
Tree 8	Base	▲	–	–	✧	–
	Bole	▲	○	○	✧	–
Tree 9	Base	–	–	–	–	–
	Bole	▲	▲	–	–	–
Tree 10	Base	▲	–	–	–	–
	Bole	–	–	–	–	▲
Tree 11	Base	▲	○	–	✧	–
	Bole	▲	–	○	–	–
Tree 12	Base	▲	▲	–	–	–
	Bole	▲	○	–	–	–
Tree 13	Base	▲	▲	–	–	–
	Bole	–	–	–	–	–
Tree 14	Base	▲	▲	–	–	–
	Bole	□	□	–	✧	▲
Tree 15	Base	○	▲	–	–	–
	Bole	□	–	○	–	–
Tree 16	Base	▲	▲	–	–	–
	Bole	▲	○	○	✧	–
Tree 17	Base	▲	▲	–	–	–
	Bole	–	–	–	–	–

Table 3. Genotypes for the tRNALeu (UAA) intron of the symbiotic *Nostoc* from 3–4 *Pectenia plumbea* thalli of the complete sampling, showing the overall photobiont diversity in the Spanish populations. Studied populations are in rows and columns represent sampled thalli. □, A1; ▲, A2; -, A5; ◊, A6.

Population	Locality name	*Pectenia plumbea* thalli			
		1	2	3	4
North-west					
I	Muniellos	□	□	◊	–
II	Moal	□	◈	◊	–
III	Monasterio de Hermo	□	□	◊	–
IV	Rebollar	□	□	□	–
Centre					
V	Gargantilla	□	▲	▲	▲
VI	El Chorro	▲	▲	▲	▲
VII	Navas de Estena	▲	▲	▲	▲
South-east					
VIII	Sierra Madrona	▲	▲	◊	–
IX	Río Mundo	□	□	□	–

Table 4. Frequency of cyanobacterial genotypes found as symbiotic (i.e. number of thalli within a lichen) and as free living (number of colonies). The upper case letter beside the name of each lichen genus match with that used in the Results to group all the genotypes of the same class. All the genotypes correspond to *Nostoc*, except 5–12 in the case of number of colonies. Genotype refers only to the nomenclature used in the study, i.e. genotypes within different

classes are distinct.

Genotype	*Nephroma* (A)		*Peltigera* (B)		*Collema* (C)	
	No. of thalli	No. of colonies	No. of thalli	No. of colonies	No. of thalli	No. of colonies
1	5	0	5	6	0	3
2	46	1	0	1	0	1
3	15	2	0	1	0	4
4	0	1	0	1	0	1
5	0	0	0	1	0	1
6	0	0	0	3	0	1
7	0	0	0	3	0	1
8	0	0	0	1	0	9
9	0	0	0	2	0	1
10	0	0	0	2	0	3
11	0	0	0	7	0	0
12	0	0	0	1	0	0

FIGURE

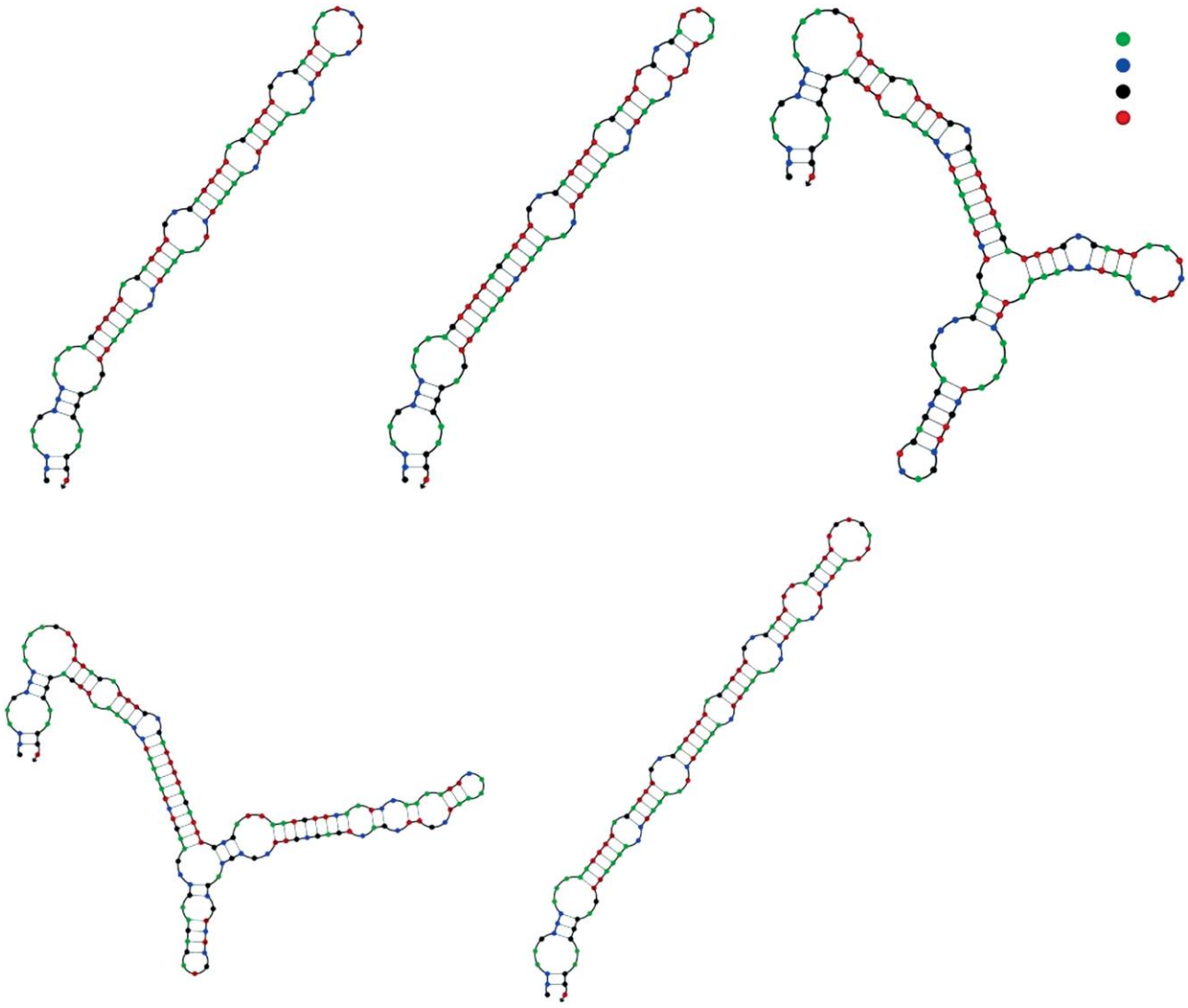

Fig. 1. Folding of the '*Nephroma* guild'-type P6b regions for the genotypes A1–A6. The genotypes A5 and A6 differ in one nucleotide but the folding yielded is identical. The genotype A4 was only found as free living. The nucleotide sequences were folded with NUPACK at 20 °C (Zadeh *et al.* 2011), and the Gibbs free energy (Δ*G*) is indicated below each stable structure.

SUPPLEMENTARY MATERIAL

Appendix 1. Detailed method description.

CULTURE MEDIA FOR MYCOBIONTS (Deason and Bold 1969; Yoshimura *et al*., 2002)

Agar medium (4% distilled water agar medium)

Agar	40 g
Distilled water	Make up to 1000 ml

MYA (Malt/Yeast Medium)

Malt extract	20 g
Yeast extract	2 g
Agar	20 g
Distilled water	Make up to 1000 ml

LBM (Lilly and Barnett's Medium)

Glucose	10 g
Asparagine	2 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$Fe(NO_3)_3 \cdot 9H_2O$	0.2 mg
$ZnSO_4 \cdot 7H_2O$	0.2 mg
$MnSO_4 \cdot 4H_2O$	0.1 mg
Thiamine	0.1 mg
Biotin	5 µg
Agar	20 g
Distilled water	Make up to 1000 ml

BBM (Basal Bold Medium)

a. Stock solutions

Flask label	Compound	Dilution of stock solution	Dilution of BBM
A	$NaNO_3$	10 g/400 ml	20 ml
B	$CaCl_2$	10 g/400 ml	10 ml
C	$MgSO_4 \cdot 7H_2O$	10 g/400 ml	10 ml
D	K_2HPO_4	10 g/400 ml	10 ml
E	KH_2PO_4	7 g/400 ml	10 ml
F	NaCl	1 g/400 ml	10 ml

b. Trace elements solutions

Flask label	Compound	Dilution of trace elements	Dilution of BBM
G	H_3BO_3	1.142 g/100 ml	1 ml
H*	$FeSO_4 \cdot 7H_2O$	0.498 g/100 ml	1 ml
	$ZnSO_4 \cdot 7H_2O$	0.882 g/100 ml	
	$MnCl_2 \cdot 4H_2O$	0.144 g/100 ml	
I*	MoO_3	0.071 g/100ml	1 ml
	$CuSO_4 \cdot 5H_2O$	0.157 g/100 ml	
	$CoNO_3 \cdot 6H_2O$	0.049 g/100 ml	
J*	EDTA	5 g/100 ml	1ml
	KOH	3.1g/100 ml	

*Several elements are mixed in one bottle.

c. BBM: Prepare stock and trace elements solutions. Autoclave and stock them in the refrigerator for future use. When BBM will be necessary, mix the amounts of the fourth column and adjust the volume with bidistilled H_2O to 1 L. Adjust the pH to 6.0.

Figure S1. A) Location of the nine studied *Pectenia plumbea* populations in Spain. B) Location of the study plot at site V (Gargantilla) where the cyanolichen and free-living cyanobacterial communities were sampled intensively (plot side length = 15 m). C) Image inside the *Arbutus unedo* forest at site V. D) Detail of an *Arbutus unedo* trunk at site V, showing the great development of some *Pectenia plumbea* thalli surrounded by the typical epiphyte lichen community.

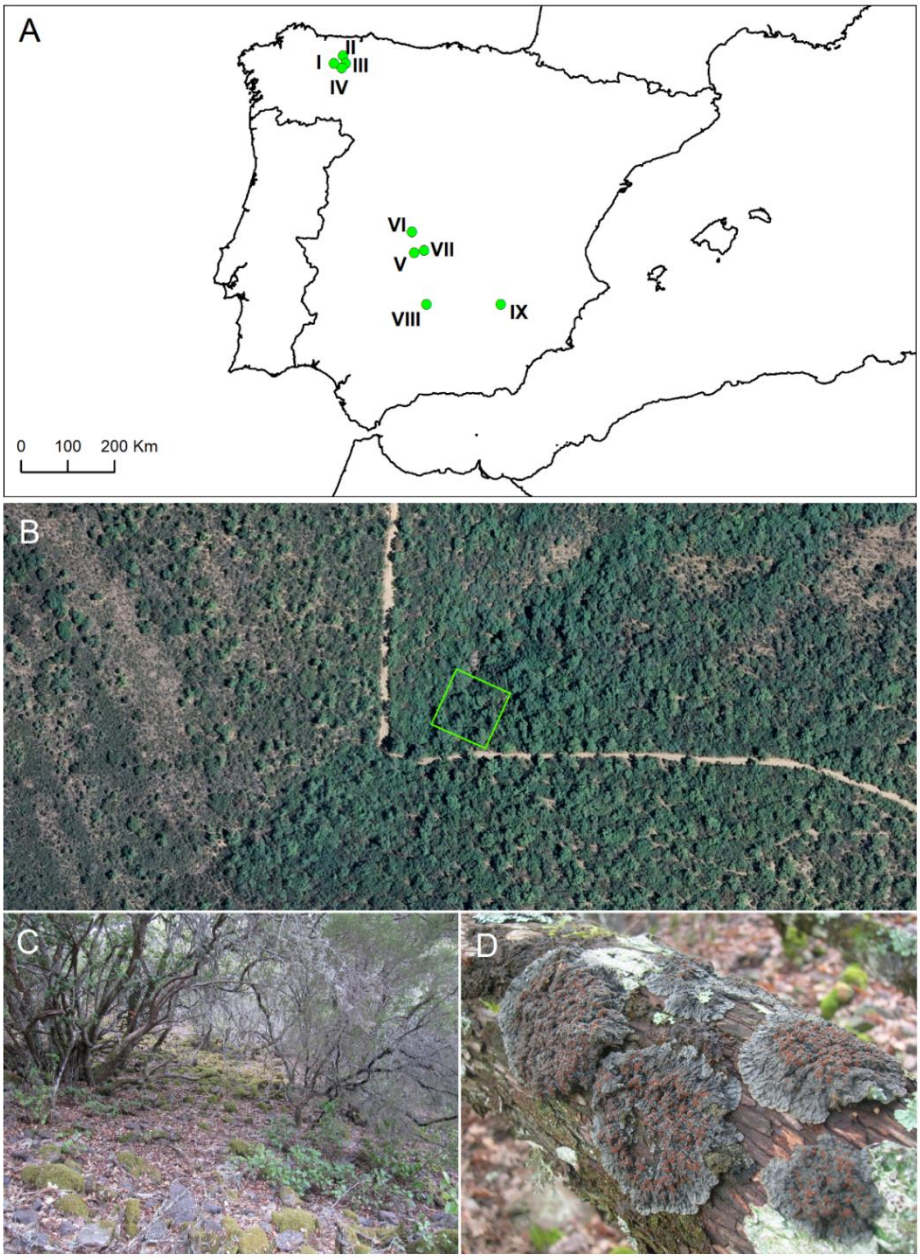

Figure S2. A–D) Representative examples of the growth of the cyanobacterial photobionts isolated from lichen thalli. Left picture was taken at the beginning of the experiment just after the isolation, and right picture was taken 50 days after the isolation. The photobionts were isolated from *Pectenia plumbea* (A and B), *Peltigera canina* (C) and *Peltigera praetextata* (D). E) and F) Detail at different scale of the profuse production of hormogonia in *Peltigera canina*. Scale bar in all pictures= 5 mm.

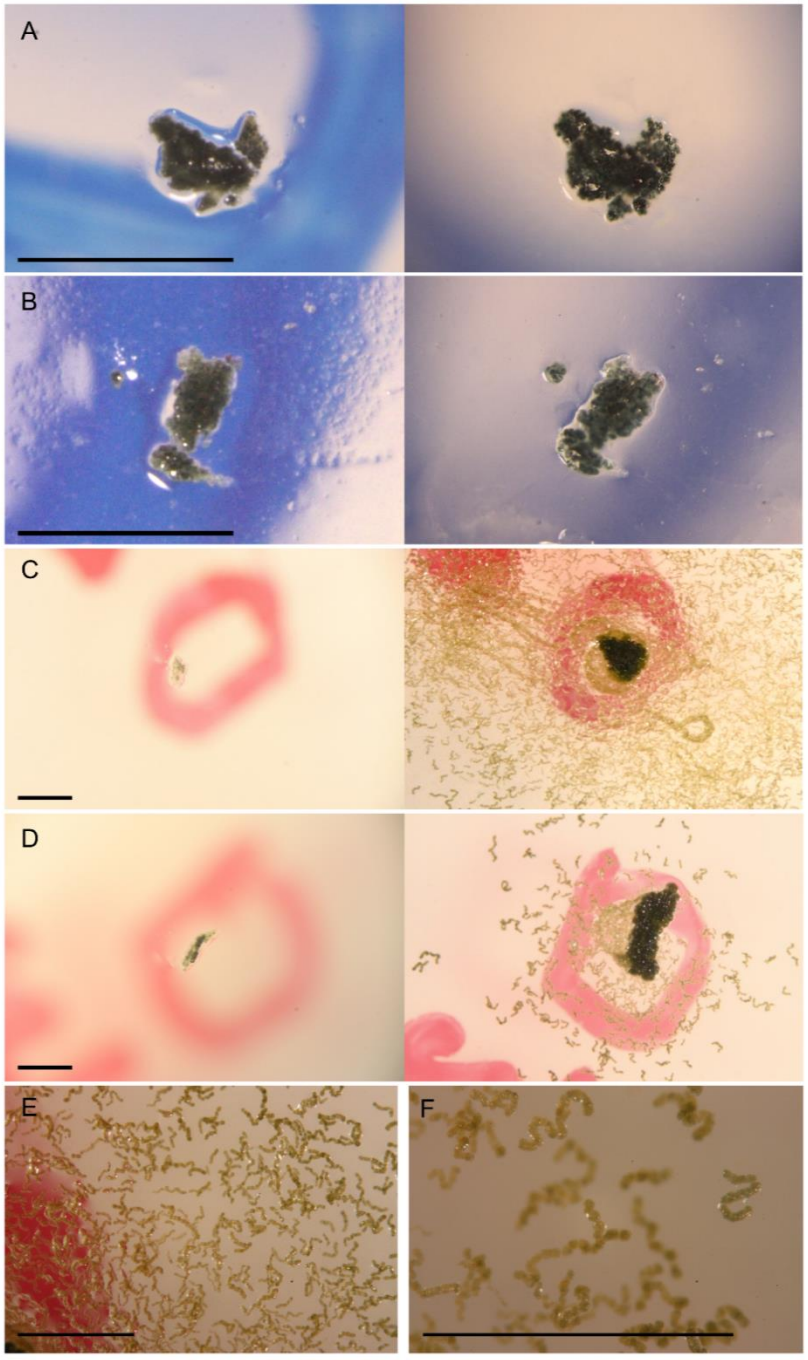

Table S1. Nucleotide sequence of the P6b region of the tRNA^{Leu} (UAA) intron of all the different *Nostoc* and cyanobacterial genotypes identified in the study, classified by the type of repeat motif. Colors highlight insertions, deletions or substitutions: fuchsia was chosen for insertions or deletions of groups of nucleotides; yellow, green, and blue indicates two, three or four different alternatives, respectively, for the same nucleotide position.

***Nephroma*-type Class I repeat motif**

Nostoc genotype A1	CAAAAGTT	TTAGATT	TGCGATT	TTAGATT	TGCGATT	AATCTTC	AATCCAA	AATTCAA	AATCTAA	AATCCAA	AATTGAG
Nostoc genotype A2	CAAAAGTT	TTAGATT	TGCGATT	TTAGATT	TGCGATT	AATCTTC	AATCCAA	AATTCAA	AATCTAA	-------	AATTGAG
Nostoc genotype A3	CAAAAGTT	TTAGATT	TGCGATT	TTAGATT	TGCGATT	AATCTTC	AATCCAA	AATTCAA	AATC TAA	AATCCAA	AATTGAG
									GTTCGACTGAGCGAAGCCGAAGTC		
Nostoc genotype A4	CAAAAGTT	TTAGATT	TGCGATT	TTAGATT	TGCGATT	AATGTTC	AATCCAA	AATTCAA	AATC TAA	AATCCAA	AATTGAG
								GTTCGACTGAGCGTTCGCGCAGCGTCTCGTAGAGAAGCCGAAGTC			
Nostoc genotype A5	CAAAAATT	TTAGATT	TGCGATT	TTAGATT	TGCGATT .	AATCTTC	AATCCAA	AATTCAA	AATCTAA	AATCCAA	AATTGAG
					TTAGATTTGTGATT						
Nostoc genotype A6	CAAAAATT	TTAGATT	TGCGATT	TTAGATT	TGCGATT .	AATCTTC	AATCCAA	AATTCAA	AATCTAA	AATCCAA	AATTCAG
					TTAGATTTGTGATT						

***Collema*-type Class I repeat motif**

Nostoc genotype B1	GAAAAAAGT	CCTGAGT	CATGAGT	GTTGAGT	GCTGAGT	AAATTTAA	AACTCAT	AACTCCT	AACTCCT	AACTCATT	AACTGTTC
Nostoc genotype B2	GAAAAAAGT	CCTGAGT	CATGAGT	GTTGAGT	GCTGAAT	AAATTTAA	AACTCAT	AACTCCT	AACTCCT	AACTCATT	AACTGTTC
Nostoc genotype B3	GAAGAAAGT	CCTGAGT	CATGAGT	GTTGAGT	GCTGAGT	AAATTTAA	AACTCTT	AACTCCT	AACTCCT	AACTCCT-	AACTGTTC
Nostoc genotype B4	GAAGAAAGT	CTTGAGT	CATGAGT	GTTGAGT	GCTGAGT	AAATTTAA	AACTCTT	AACTCCT	AACTCCT	AACTCCT-	AACTGTTC
Cyano genotype B5	GAAGAAAGT	AGTAAGT	GCTGAGT	CATGAGT	CCTGAGT	AAATTAAA	AACTCAT	AACTCCT	AACTCCT	AACTCCT-	AAAAATTC
Cyano genotype B6	GAAGAAAGT	AGTAAGT	GCTGAGT	CATGAGT	CCTGAGT	AAATTAAA	AACTCAT	AACTCAT	-------	AACTCCT-	AAAAATTC
Cyano genotype B7	GAAAAAAGT	CGTGAGT	GCTGAGT	GGCCAGT	TGTGAGT	AAAT-TAA	AACTCTT	AACTCTT	AACTCCT	AACTCCT-	AACTATTC

Cyano genotype B8 GAAAAAAGT CGTGAGT AATGAGT ACTGAGT ACTGAGT AAATTAAA A-CTCCT AACTCTT AACTCCT AACTCCT- AACTATTC

Cyano genotype B9 GAAAAAAGT CGTGAGT AATGAGT ACTGAGT ACTGAGT AAATTAAA A-CTCCT AACTCCT ACACTCCT AACTCCT- AACTATTC

Cyano genotype B10 GATAAGAGT GCTGAGT GCTGAGT GATGAGT GATGAGT TAAAAGTT AACTCCT AACTTCT ACTTTGT -TCTCCT- AACTCTTC

Cyano genotype B11 GAAGAAAGT TCTGAGT AGTGAGT ------- GGTGAGT AAACCAAA AACTCCT AACTCCT ------- AACTC-TT AACCAATC

Cyano genotype B12 CAAA- AATG TCCGAGT CATGAGT GTTGAGT GCTCAAA AAATTTAA AACTCAT AACTCCT AACTCCT AACTCATT AACTGTGC

***Peltigera*-type Class II repeat motif**

Nostoc genotype C1 CAAAAATT TTAGATT TGTGATT TTAGATT TGCGATT AGTCTTC AATCCAA ------ ------ AATCCAA AATTGCG

Nostoc genotype C2 CAAAAATT TTAGATT TGCGATT TTAGATT TACGATT AGTCTTC AATCCAA AATTCAA ------ ------ AATTGAG

Nostoc genotype C3 CAGAAATT TTAGATT TGCGATT TTAGATT TACGATT AGTCTTC AATCCAA AATTCAA AATCTAA AATCAAA AATTGAG

Nostoc genotype C4 CAAAAATT TTAGATT TGTGATT TTAGATT TACGATT AGTCTTC AATCCAA AATCCAA AATC-TAA AATCCAA AATTGCG
GTTCGACTGAGCAAAGCCGAAGTC

Nostoc genotype C5 CAAAAATT TTAGATT TGCGATT TTAGATT TGCGATT AGTCTTA ------ AATTCAA AATC-TAA AATCCGG AATTGAG
GTTCGACTGAGCGTTCGCGCAGCGTCTCCTAGAGAAGCCGAAGTC

Nostoc genotype C6 CAAAAATT TTAGATT TGTGATT ------ ------ AGTGTTC AATCCAA AATCCAA AATC-TAA AATCCAA AATTGCG
GTTCGACTGAGCCAAGCTGAAGTC

Nostoc genotype C7 CAAAAATT TTATATT TGCGATT ------ ------ AGTCTTT AAACCAA AATTCTA AATC-TAA AATCCAA AATTGAG
GTTCGACTGACCGAAGCCGAAGTC

Nostoc genotype C8 CAAAAATT TTAGATT TGTGATT ------ ------ AGTGTTT AATCCAA AATTCAA AATC-TAA AATCCAA AATTGAG
GTTCGACTGAGCGAAGCCGAAGTC

Nostoc genotype C9 CAAAA-TT TGAGATT TGCGA-TT TGAGATT TGCGATT AGTCTTC AATCCAA AATCCAA AATC-TAA AATCCAA AATTGCG
TAGCGTAGCGTTAGCGACGAAGGAGCGTC GTTCGACTGAGCCAAGCCGAAGTC

Nostoc genotype C10 CAAAA-TT TAAGATT TGCGA-TT TGAGATT TGCGATT AGTCTTC AATCCAA AATCCAA AATC-TAA AATCCAA AATTGCG
TAGCGTAGCGTTAGCGACGAAGGAGCGTC GTTCGACTGAGCCAAGCTGAAGTC

Table S2. Summary of a previous survey (Aragón, unpublished results) of 588 plots along the Spanish Mediterranean region, showing the 26 plots where *Pectenia plumbea* was present. The values of abundance of *Pectenia plumbea, Dendriscocaulon umhausense* and *Fuscopannaria mediterranea* are expressed in the percentage of surface occupied in the sample grids. Phorophyte is the tree species were the lichen inventory was carried out.

Phorophyte	Forest fragment code	Plot number	*P. plumbea* abundance	*D. umhausense* abundance	*F. mediterranea* abundance
Quercus pyrenaica	AY-15	1	15	13	9
Quercus pyrenaica	MT-01	1	5	15	15
Quercus pyrenaica	MT-01	2	4	15	15
Quercus pyrenaica	MT-08	1	2	0	4
Quercus pyrenaica	SM-01	1	5	13	11
Quercus pyrenaica	SM-04	1	6	11	15
Quercus pyrenaica	SM-06	1	8	9	11
Quercus pyrenaica	SM-06	2	9	12	14
Quercus pyrenaica	SM-09	1	5	9	13
Quercus pyrenaica	SM-10	1	8	9	11
Quercus pyrenaica	SB-01	1	7	10	7
Quercus pyrenaica	SC-1	1	2	2	6
Quercus pyrenaica	SC-2	1	1	3	8
Quercus faginea subsp. *broteroi*	SM-11	1	2	7	9
Quercus faginea subsp. *broteroi*	SM-15	1	5	9	13
Quercus faginea subsp. *broteroi*	SM-19	1	7	13	15
Quercus faginea subsp. *broteroi*	MT-19	1	9	12	15
Quercus faginea subsp. *broteroi*	MT-21	1	7	14	15
Quercus faginea subsp. *broteroi*	MT-21	2	9	12	12
Quercus ilex subsp. *ballota*	59	1	6	8	13
Quercus ilex subsp. *ballota*	78	1	4	7	13
Quercus ilex subsp. *ballota*	82	1	3	7	14
Quercus ilex subsp. *ballota*	92	1	3	7	10
Quercus ilex subsp. *ballota*	94	1	10	10	14
Quercus ilex subsp. *ballota*	97	1	8	11	13
Quercus ilex subsp. *ballota*	102	1	7	9	14