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Substrates of *Peltigera* Lichens as a Potential Source of Cyanobionts

Catalina Zúñiga[1] · **Diego Leiva**[1] · **Margarita Carú**[1] · **Julieta Orlando**[1]

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Abstract Photobiont availability is one of the main factors determining the success of the lichenization process. Although multiple sources of photobionts have been proposed, there is no substantial evidence confirming that the substrates on which lichens grow are one of them. In this work, we obtained cyanobacterial 16S ribosomal RNA gene sequences from the substrates underlying 186 terricolous *Peltigera* cyanolichens from localities in Southern Chile and maritime Antarctica and compared them with the sequences of the cyanobionts of these lichens, in order to determine if cyanobacteria potentially available for lichenization were present in the substrates. A phylogenetic analysis of the sequences showed that *Nostoc* phylotypes dominated the cyanobacterial communities of the substrates in all sites. Among them, an overlap was observed between the phylotypes of the lichen cyanobionts and those of the cyanobacteria present in their substrates, suggesting that they could be a possible source of lichen photobionts. Also, in most cases, higher *Nostoc* diversity was observed in the lichens than in the substrates from each site. A better understanding of cyanobacterial diversity in lichen substrates and their relatives in the lichens would bring insights into mycobiont selection and the distribution patterns of lichens, providing a background for hypothesis testing and theory development for future studies of the lichenization process.

Keywords Cyanobacteria · *Nostoc* · *Peltigera* · Photobiont availability · Terricolous lichens

Introduction

Since the late 1860s, lichens have been classically defined as stable symbiotic associations between an ascomycete fungus (mycobiont) and at least one photoautotrophic component (photobiont), consisting of a green alga or a cyanobacterium. However, recently, this classical description was updated when a new symbiont was described in various lichens, corresponding to basidiomycete yeasts [1]. Before this new symbiont was discovered, other microorganisms had also been found in close association with the lichen thallus, of which the most researched correspond to bacteria [e.g., 2–6]. Indeed, lichens have changed from being described as bi-partite or tri-partite organisms to multispecies symbioses [7]. Nevertheless, even when only the classical components are considered, the ecological and genetic factors determining lichenization, the term used to describe the development of a successful lichen symbiosis, are still poorly understood.

During horizontal transmission of a lichen-forming fungus, the released fungal spore must find a potential photobiont with which to establish symbiosis. However, even after vertical transmission, i.e., symbiont co-dispersal, the mycobiont may substitute its photobiont with a more suitable partner in a process called photobiont switching [8]. Therefore, in both forms of transmission, photobiont availability is a key factor determining the success of the lichenization process. If there is

Catalina Zúñiga and Diego Leiva contributed equally to this work and are considered joint first authors.

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✉ Julieta Orlando
jorlando@u.uchile.cl

[1] Laboratory of Microbial Ecology, Department of Ecological Sciences, Faculty of Sciences, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile

no suitable photobiont available, most lichen-forming fungi are not able to survive in the free-living state [9].

Several strategies have been proposed that might provide lichen-forming fungi with photobionts to reconstitute a lichen symbiosis from one generation to the next. These include extracting photobiont cells from the thalli of other lichens [10] or from other organisms [11, 12] or temporarily persisting in association with incompatible photobionts [13] or in a free-living state [14] until a compatible photobiont is encountered. Most studies have addressed availability by comparing the photobionts of each lichen species with the pool of photobiont genotypes represented by co-occurring lichens or other organisms symbiotically interacting with photobionts at the same locations [9, 10, 12, 15]. However, these are not the only sources of photobionts for the generation of new associations, since they could also be obtained from aposymbiotic populations [16].

Though several studies reporting the lichen microbiome have been performed, none have considered it as a source of potential photobionts [e.g., 2–6], and only two assessed the lichen thallus surface as an environment of potential photobionts [17, 18]. Also, frequent lichen substrates like bark [19, 20] or rocks [21] have been evaluated as photobiont habitats, concluding that these environments represent potential temporary niches for free-living stages of lichen photobionts. However, to the best of our knowledge, potential photobionts from the substrates of terricolous lichens, i.e., lichens growing on soil as a substrate, have not been evaluated. Therefore, the possible connections between potential photobionts in the lichen thalli and their relatives in the lichen's surroundings remain almost unknown [22].

The selection of a suitable photobiont among those available is a key factor in the development of lichens, although there is still some controversy shrouding this process [23]. Some authors concluded that this factor is mainly dependent on the taxonomy of the mycobiont, as the same taxa have been found to be associated with restricted groups of photobionts across considerable habitat boundaries [24–26]. On the other hand, several reports have shown that selectivity also depends on the characteristics of the habitat, with mycobionts showing lower selectivity towards their photobionts under extreme conditions [27–30]. Furthermore, others have concluded that photobionts exhibit clear preferences for environmental factors, limiting the ecological niches available to lichens and leading to the existence of photobiont-mediated guilds, forming a common pool of horizontally linked photobionts [12, 31–33]. This suggests that a mix between low availability of photobionts and limiting environmental factors would favor more versatile (less selective) mycobionts [34, 35]. Therefore, the variation among lichen photobionts would be mainly the result of an evolutionary selection within and between ecological habitats [19, 36].

It is clear then that patterns of lichen distribution may be influenced by many interacting factors, which include symbiont availability, reproductive strategy, abiotic environment, and the specific ecological requirements of each symbiont [15]. Here, we determined the presence of cyanobacteria potentially available for lichenization with terricolous *Peltigera* lichen-forming fungi in localities from Southern Chile and maritime Antarctica, regions that remain poorly studied in lichenological terms [37–40]. Given that the cyanobacterial diversity in lichen substrates and the possible overlap of this diversity with that in lichens are almost unknown, we compared the diversity of (i) the cyanobionts lichenized with *Peltigera* cyanolichens and (ii) the potential cyanobionts present in the substrates of these lichens, considering both sources as potential reservoirs of lichen cyanobionts.

Methods

The samples for this study consisted of 186 *Peltigera* cyanolichens plus the substrates directly underneath them, which corresponded to soil alone or associated with plants (liverworts, mosses, angiosperms, among others). Samples were obtained from nine sites covering four localities in Southern Chile and maritime Antarctica (Table 1 and Fig. 1). All lichens had been previously classified by Zúñiga et al. [40] into eight groups of mycobionts (M1–M8) according to the phylogenetic analyses of their 28S ribosomal RNA (rRNA) gene: *Peltigera ponojensis* (M1), *Peltigera extenuata* (M2), *Peltigera* sp. (M3), *Peltigera rufescens* (M4), *Peltigera canina* lineage (M5), *Peltigera frigida* (M6), *Peltigera neckeri* lineage (M7), and *Peltigera hymenina* lineage (M8).

The substrate superficially associated with the collected portion of each thallus was removed with a brush and a spatula. In order to decrease spatial heterogeneity and generate a reasonable number of samples for clone libraries, 100 mg of each substrate sample (Table 1) were combined into a single composite sample per site. For example, 100 mg of each of the 20 substrate samples from Karukinka young forest (KY) was combined into a single KY composite sample. DNA was extracted from 250 mg of each composite sample with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., CA, USA) according to the manufacturer's instructions. The quality and integrity of the extracted DNA were visualized in 0.8% (*w*/*v*) agarose gels in TAE 1× buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) stained with GelRed™ (Biotium, CA, USA).

For each of the nine composite samples, 16S rRNA gene amplicons were obtained with primers *Cya*106F-*Cya*781R, which were originally developed for cyanobacteria and plastids [41]. These primers do not amplify the entire 16S rRNA gene but include regions v2, v3, and most of v4, giving a final amplicon size of ~650 bp. PCR mixes were prepared using

Table 1 Sampling localities and sites from Southern Chile and maritime Antarctica

Localities	Sites	Latitude	Longitude	Altitude (m a.s.l.)	Number
Coyhaique (C)	CF1	−45.5276	−72.0342	709.2	26
	CF2	−45.5297	−72.0278	705.4	25
Karukinka (K)	KM	−54.1270	−68.7094	169.3	20
	KY	−54.1397	−68.7101	186.4	20
	KG	−54.1263	−68.7088	153.4	20
Navarino (N)	NM	−54.9484	−67.6534	88.4	20
	NY	−54.9391	−67.6028	38.4	20
	NG	−54.9407	−67.6291	33.7	20
Deception (D)	DH	−62.9728	−60.5757	20.6	15

CF1 Coyhaique forest 1, *CF2* Coyhaique forest 2, *KY* Karukinka young forest, *KM* Karukinka mature forest, *KG* Karukinka grassland, *NY* Navarino young forest, *NM* Navarino mature forest, *NG* Navarino grassland, *DH* Deception hillside

GoTaq® Green Master Mix (GoTaq® DNA polymerase in 1× Green GoTaq® Reaction Buffer [pH 8.5], 200 μM of each dNTP, and 1.5 mM $MgCl_2$) (Promega, WI, USA) and amplified in a Maxygene thermocycler (Axygen, CA, USA). The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplicons were cloned and sequenced by the Library Construction and Sequencing Service provided by Macrogen (Macrogen Inc., Seoul, South Korea), generating a total of nine clone libraries with 96 sequenced clones each.

DNA sequences were visually checked and manually edited using Mega v5.2 software [42] and aligned with the Muscle alignment tool [43] provided in the same software. Ambiguously aligned nucleotides were checked on the web server Guidance [44] and removed prior to the subsequent analyses. Edited sequence fragments were subjected to BLASTn queries [45] for an initial verification of their identities by comparison with the non-redundant nucleotide database at GenBank (NCBI). Chloroplast sequences were broadly classified as liverworts, mosses, green algae, angiosperms, ferns, or diatoms (Online Resources Table S1). Cyanobacterial sequences were uploaded to GenBank database under accession numbers KX255064 to KX255351 (Online Resources Tables S2 and S3) and subjected to phylogenetic analyses.

Cyanobacterial phylotypes were defined using a 99.7% cutoff based on a sequence identity matrix in BioEdit v7.2.5 [46]. Then, a sequence set was built with one representative of each cyanobacterial phylotype from the substrates, one representative of each of the lichenized *Nostoc* cyanobionts from Zúñiga et al. [40], and 70 cyanobacterial 16S rRNA gene sequences from GenBank for comparison. The latter consisted of a selection of the *Nostoc* sequences reported by O'Brien [47], in addition to close matches to our sequences according to the BLASTn results, in case they were not already included in that study. *Gloeobacter violaceus* PCC 7421 (accession number NR074282) and *Pseudanabaena* sp. PCC 7367 (accession number NR102446) were set as outgroups.

These sequences were submitted to maximum likelihood (ML), Bayesian inference (BI), and neighbor joining (NJ) phylogenetic reconstructions. The best nucleotide substitution model was determined with the help of jModelTest v2.1.6. [48, 49] under the CIPRES portal v3.3 [50], using the corrected Akaike information criteria (AICc), which suggested TPM2uf+I+G as the best fitting model of evolution for the ML analyses. In the subsequent phylogenetic analyses, the GTR+I+G model was used instead, as it was the closest available in all platforms.

ML analysis was performed on the T-REX web server [51] under the PhyML algorithm [48] using 1000 bootstrap repetitions for support. BI was carried out using the Metropolis-coupled Bayesian Markov chain Monte Carlo algorithm $(MC)^3$ implemented in the software MrBayes v3.2.2 [52] in the CIPRES portal v3.3 [50]. Four independent runs of five million generations each were made, sampling the chains every 1000th generations. The first 2500 samples were discarded as burn-in, and the convergence of the chains was assessed using Tracer v1.6 [53]. NJ was carried out using FastME v2.0 software [54] under the ATGC South of France bioinformatics platform (http://www.atgc-montpellier.fr/) using 1000 bootstrap repetitions for support. The ML phylogenetic tree was drawn on TreeGraph v2.9.1-617 beta [55].

Finally, in order to evaluate whether a suitable number of sequences were considered, rarefaction and coverage analyses were performed. The first was assessed using the observed number of phylotypes in the software EstimateS [56] and adjusting the data to a theoretical curve by non-linear regression, in order to obtain the theoretical number of phylotypes (GraphPad Prism 4.0). The second was calculated using the coverage index $Cx = 1 - (N_x / n)$, where N_x is the number of phylotypes and n is the total number of individuals.

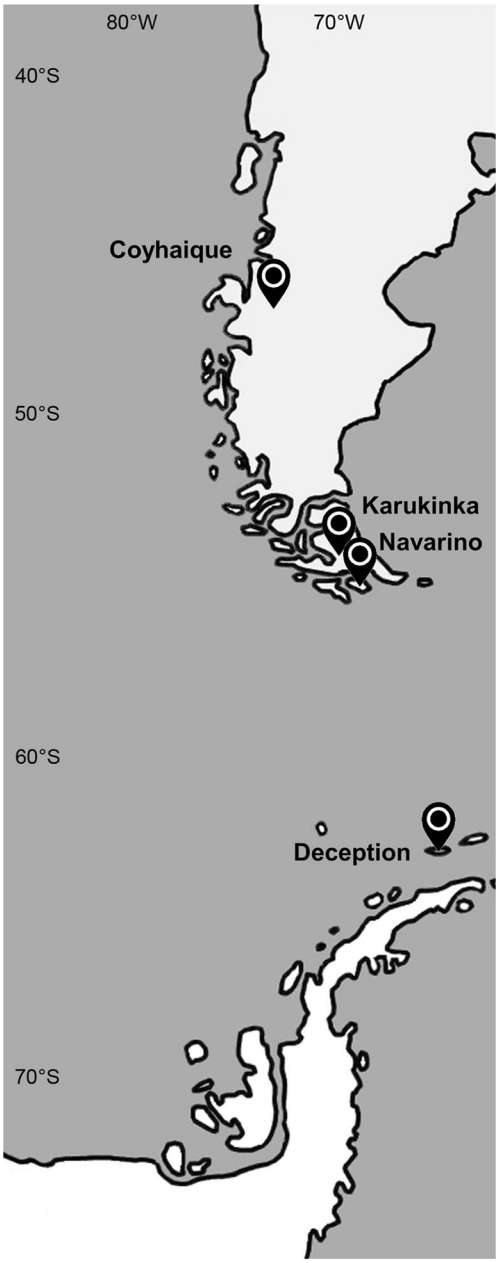

Fig. 1 Map of the sampling localities from Southern Chile and maritime Antarctica

Results

A total of 864 16S rRNA gene sequences were obtained from the nine composite samples (Table 2). Among them, 53.2% (460 sequences) corresponded to chloroplast sequences (Table 2) which were mainly related to liverworts, mosses, green algae, and in some cases to angiosperms, ferns, and diatoms (Online Resources Table S1). The detection of these chloroplast sequences in the substrates of lichens was concordant with the presence of the related organisms at each site.

The cyanobacterial sequences represented 33.3% (288 sequences), and their phylogenetic analysis showed that different genera, such as *Tolypothrix*, *Scytonema*, and *Microcoleus* were found throughout the sampled substrates. However, *Nostoc* was by far the dominant genus, with an abundance of 274 out of 288 sequences (95.1%), and a broad diversity, comprising 95 out of 106 phylotypes (89.6%) (Table 3 and Online Resources Fig. S1).

To determine which of the sequences recovered from the substrates corresponded to potential cyanobionts, a comparison was made between their 16S rRNA gene nucleotide sequences and the ones of the lichenized *Nostoc* cyanobionts included in Zúñiga et al. [40]. By an operational definition proposed in this study, only those that possessed 99.7% sequence identity with the lichenized cyanobionts were considered as a positive match. This apparently strict identity criterion was chosen because when using a marginally lower identity value (99.6%), it was not possible to assign most of the sequences to just one cyanobiont type, but it, in turn, did allow the consideration of one or two possible polymerase errors. Since the primers used to obtain sequences from the substrates amplified a shorter fragment of the 16S rRNA gene than those used for the lichenized cyanobionts in Zúñiga et al. [40], phylotypes AC13 and AC14 (previously C13 and C14, respectively) could not be distinguished. Therefore, the sequences of these phylotypes were assigned to the corresponding lichenized cyanobionts in the same proportion as their abundances at each site.

From the total *Nostoc* sequences, available potential cyanobionts in the substrates appear to be much more abundant than the non-symbiotic cyanobacteria, comprising on average 60.7% of the abundance, although only 19.3% of the richness

Table 2 Abundance of the clone sequences at each site

Sites →	Total	CF1	CF2	KM	KY	KG	NM	NY	NG	DH	Average
Cyanobacteria (*Nostoc*)	288 (*274*)	38 (*37*)	27 (*27*)	20 (*20*)	31 (*31*)	6 (*3*)	42 (*42*)	36 (*34*)	54 (*47*)	34 (*33*)	32.0 (*30.4*)
Chloroplasts	460	40	54	58	48	83	46	54	32	45	51.1
Chimeras	92	18	9	9	12	4	8	5	10	17	10.2
Others	24	0	6	9	5	3	0	1	0	0	2.7

The numbers in parentheses indicate the abundance of *Nostoc*

CF1 Coyhaique forest 1, *CF2* Coyhaique forest 2, *KY* Karukinka young forest, *KM* Karukinka mature forest, *KG* Karukinka grassland, *NY* Navarino young forest, *NM* Navarino mature forest, *NG* Navarino grassland, *DH* Deception hillside

Table 3 Diversity of *Nostoc* sequences at each site

Sites →	Total	CF1	CF2	KM	KY	KG	NM	NY	NG	DH	Average
Nostoc abundance	274	37	27	20	31	3	42	34	47	33	30.4
SC[a] abundance	186	20	18	13	25	0	32	23	34	21	20.7
LC[b] abundance	186	26	25	20	20	20	20	20	20	15	20.7
Nostoc types	95	17	12	11	7	3	13	15	18	12	12.0
SC types	12	1	3	4	2	0	2	4	5	1	2.4
LC types	14	4	3	6	4	4	3	4	5	1	3.8

CF1 Coyhaique forest 1, *CF2* Coyhaique forest 2, *KY* Karukinka young forest, *KM* Karukinka mature forest, *KG* Karukinka grassland, *NY* Navarino young forest, *NM* Navarino mature forest, *NG* Navarino grassland, *DH* Deception hillside

[a] SC: available potential cyanobionts in the substrates

[b] LC: available lichenized cyanobionts in the thalli

(Table 3). In fact, the coverage of the theoretical diversity reached 0.65 for *Nostoc* (329 theoretical phylotypes, R^2 = 0.9968), while for the potential cyanobionts in the substrates it was 0.94 (12 theoretical phylotypes, R^2 = 0.9826). The low richness of available potential cyanobionts in the substrates was not correlated with the high richness of *Nostoc* (R^2 = 0.3097; p = 0.1197), as can be seen, for example, in Coyhaique forest 1 (CF1), where 17 different *Nostoc* types were found (Table 3), but only one of the cyanobacteria in the substrates was related with a cyanobiont detected also in the lichens [40]. Additionally, the substrate from Deception hillside (DH), despite having just one available potential cyanobiont phylotype, presented an average richness of *Nostoc* (12).

The abundance of the lichenized and potential cyanobionts retrieved from the substrates was positively correlated (R^2 = 0.4466; p = 0.0090). In other words, in most cases, the most abundant cyanobionts in the lichens were also those with the highest abundance in the substrates. The abundance of both the lichenized and potential cyanobionts from substrates was considered as the abundance of the available potential cyanobionts (AC) in the different sites and is shown with different textures in Fig. 2. In some cases, the abundance of the available cyanobionts was higher in the lichens than in the underlying substrates (Fig. 2, textured vs. black bars), with AC4 and AC9 as extreme cases since they were not even detected in the substrates. However, other ACs were more than threefold more abundant in the substrates than in the lichens, such as AC5 in Karukinka young forest (KY) and AC3 in Navarino grassland (NG).

Even though some ACs were mainly found in a certain sampling site (e.g., AC1), others were common to more than one site; among them, AC14 was the most widely distributed cyanobiont. In fact, AC14 was present in six out of the nine sites in all four sampled localities (Fig. 2), and it was also the one associated with the highest number of different mycobionts (Fig. 2, AC14 has the most diverse pattern of textured bars). Within the sampling localities, AC14 was considerably more abundant in DH and NG.

Although it was not a general pattern, several cyanobionts were associated with two or more distinct mycobionts in each site. The most extreme cases of this were AC3 in Coyhaique forest 2 (CF2, associated with four different mycobionts) and AC2 in Karukinka mature forest (KM, associated with three different mycobionts) (Fig. 2). Conversely, mycobiont M8 was exclusively associated with AC10, AC11, and AC12, which, in turn, were not associated with other mycobionts.

Finally, it is worth noticing that in most sites, only representatives of the lichenized cyanobionts were detected in the substrates (except for one sequence in Navarino young forest (NY) and three sequences in NG), i.e., if a cyanobiont was not present in the lichens from a determined site, it was also not detected in their respective substrates (Fig. 2).

Discussion

Cyanobacterial populations from the substrates underlying 186 terricolous *Peltigera* cyanolichens from localities in Southern Chile and maritime Antarctica were evaluated and compared with the cyanobionts of these lichens, in order to determine if cyanobacteria potentially available for lichenization were present in the substrates. The molecular marker chosen (16S rRNA gene) responds to the need of comparing the sequences obtained from the lichen substrates with those from the lichen thalli published by Zúñiga et al. [40]. Even though the primers used in the present study do not amplify the entire 16S region, they were chosen given their specificity for cyanobacteria [41] and because they still amplify a substantial portion of the gene, providing sequences suitable for phylogenetic analyses. Since the communities from our study were vastly dominated by a single taxon (*Nostoc*, 95.1%) and the diversity of potential cyanobionts in the substrates was highly covered (Cx = 0.94), cloning and Sanger sequencing are cost-effective tools for obtaining broad overviews of the diversity of potential cyanobionts in the substrates, avoiding the challenge of extracting information from

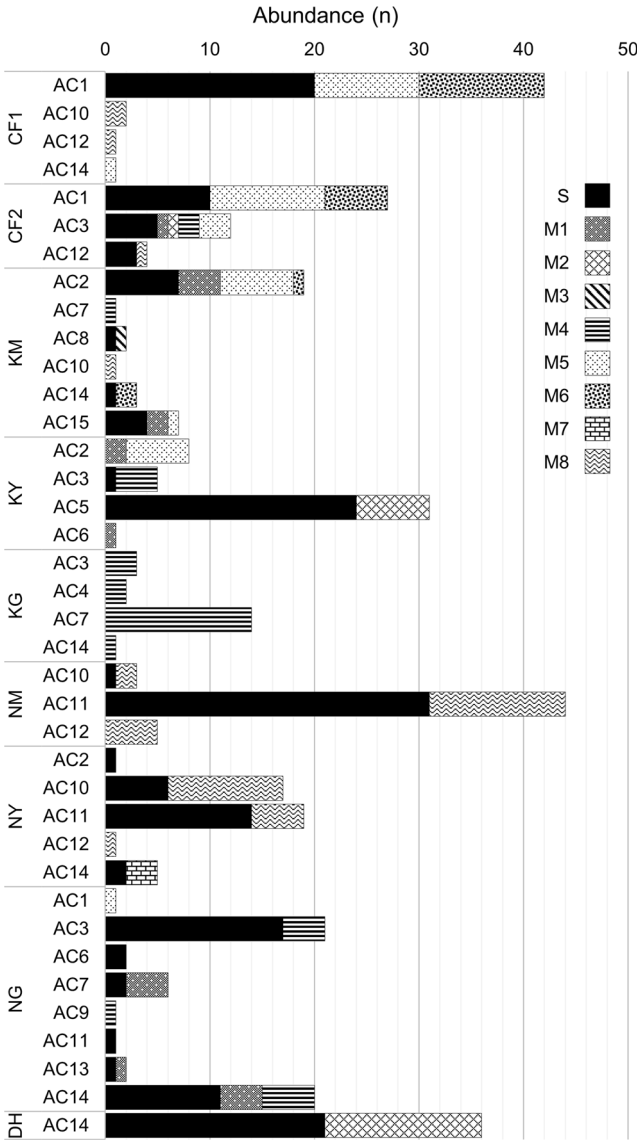

Fig. 2 Cyanobiont availability per site. Available cyanobiont phylotypes (*AC1–AC15*), including the lichenized ones associated with mycobiont phylotypes (*M1–M8*) (*textured bars*) and the potential cyanobionts in the substrates (*S*) obtained in this work (*black bars*). *CF1* Coyhaique forest 1, *CF2* Coyhaique forest 2, *KY* Karukinka young forest, *KM* Karukinka mature forest, *KG* Karukinka grassland, *NY* Navarino young forest, *NM* Navarino mature forest, *NG* Navarino grassland, *DH* Deception hillside. *P. ponojensis* (*M1*), *P. extenuata* (*M2*), *Peltigera* sp. (*M3*), *P. rufescens* (*M4*), *P. canina* lineage (*M5*), *P. frigida* (*M6*), *P. neckeri* lineage (*M7*) and *P. hymenina* lineage (*M8*)

high-throughput sequencing data sets [57]. Using DNA purified from substrates underneath lichens, we obtained 864 clones, which in most cases were sufficient to recover sequences related to those retrieved from the lichenized *Nostoc* phylotypes at each site and to provide insights into which of these phylotypes would be the most abundant in the DNA isolated from the substrates.

Regarding the distribution of *Nostoc* phylotypes, Magain et al. [35] proposed that it reflects their level of specialization, with generalists covering more extensive geographical distributions than specialists. Overall, our results are in accordance with Magain et al. [35]. For example, AC1 was associated with only two mycobionts, and it was highly abundant only in Coyhaique, being present in only one lichen elsewhere (Navarino) [58]. In addition, AC10, AC11, and AC12 were only present in two forested sites (CF1 and KM) and were associated with M8, the only mycobiont described in our sampling sites as belonging to the Polydactylon section of *Peltigera* [40], thus reflecting a phylogenetic specificity of symbiont pairs. Conversely, AC14 was associated with six mycobionts; it was not only present in all localities sampled but it was also markedly more abundant in those that could be considered the most extreme sites in this study, given their high latitudes and unforested characteristics: the southernmost grassland and the Antarctic hillside (NG and DH, respectively) [40]. This suggests that AC14 is versatile and capable of adapting to different ecological conditions, even when it was not associated with any of the reference sequences analyzed in the phylogeny. Although sequences from multiple origins were included in the analysis, only a few were available from South America [e.g., 59–61], which might be more a reflection of the scarcity of studies from this region than a genuine biogeographical pattern per se.

Two observations are worth highlighting with respect to the diversity of *Nostoc* in the environments studied. Firstly, the diversity of *Nostoc* between the two grasslands (KG vs. NG) was unexpectedly different, considering that photobiont availability is a factor that is directly influenced by environmental conditions [28, 31, 62], and these two sites possess similar ecological characteristics. However, these differences could reflect the conspicuous presence of herbaceous plants (mainly Asteraceae) cohabiting with lichens in KG; although the substrate samples consisted of the soil closely adhered to the lichen thalli, the presence of plant material in high abundance may have decreased the amplification from cyanobacteria. Secondly, the Antarctic site (DH) presented an average richness of *Nostoc*, even though lower values might be expected in such extreme conditions. Even though it is known that cyanobacteria are scarce in the most extreme habitats of Antarctica [63, 64], our results agree with studies of cyanobacteria in maritime Antarctica that report a broad diversity of *Nostoc* strains, both free-living and symbiotically associated with lichen-forming fungi or bryophytes [28]. This is likely explained by their physiological versatility and ample ecological tolerance, allowing them to compete successfully with other organisms in aquatic and terrestrial environments [65].

Although no clear guild structures could be reported from our sampling, some cyanobionts were shared by several mycobionts in a determined sampling site, suggesting the possibility of horizontal symbiont transfer in lichens [19]. Indeed, some studies have found that these guild structures are

frequently found when communities of organisms associated with photobionts co-exist [10, 12], and it has been proposed that these photobiont-mediated guilds could have an important role in the evolution of symbiotic organisms [19]. Recently, Manoharan-Basil et al. [66] described a new species of *Peltigera* which presented cyanobionts related to those associated with co-existing species. Although our sampling included a potential new species (M3) [40], it was related to a cyanobiont (AC8) that was not associated with any other mycobiont included in our sampling sites. In any case, it is important to keep in mind that many of the *Nostoc* sequences that were not considered potential cyanobionts, according to the operational definition adopted in this study, might still be potential cyanobionts of other *Peltigera* species that were outside the limits of our sampling.

Interestingly, there was considerable overlap in the cyanobiont phylotypes of lichen and substrate samples; i.e., in general, the most abundant phylotypes identified from lichen specimens of a particular site were also the most abundant in the substrates from the same site. Conversely, most of the cyanobionts that were not present in the lichens from a determined site were also not detected in their respective substrates. One explanation is that these cyanobacteria originate from the lichens growing in that site through specialized lichen propagules such as soredia and isidia, through lichen fragments, or even escaping from the symbiotic thallus to a free-living stage in the substrate, since lichen thalli are a recognized source of photobionts in horizontal transmission or in photobiont switching [67, 68]. The alternative explanation is that, given their presence in the substrate, these specific cyanobionts were the only ones available for lichenization, therefore explaining their occurrence in symbiosis. These alternatives are not mutually exclusive, and further studies need to be performed in order to determine the most likely explanation.

Since the number of *Nostoc* sequences obtained from the substrates was greater than that obtained from the lichens, it is noteworthy that in most sites, the abundance of the available cyanobionts was higher in the lichens than in the underlying substrates. A possible explanation for this apparent rarity in the substrates could mean that cyanobacteria have a greater fitness in symbiosis than as free-living organisms [69]. However, their low abundance also in the lichens might indicate that these were in fact present in the substrates, but below detectable levels, considering that the DNA extracted from the lichens has a higher abundance of the DNA of their own cyanobionts, while the DNA from the substrates also contains other cyanobacterial genomes competing for amplification. In the case of those phylotypes that were present in lichens but not detected in the substrates, horizontal transmission of the photobiont through thallus fragmentation or vegetative propagules might be a possible explanation [35]. Conversely, three ACs were found in the substrates but not in the lichens of two sites in Navarino, which is in accordance with the proposition that *Peltigera* mycobionts would not associate with all *Nostoc* phylotypes present in a specific locality [35].

Observational studies (i.e., any quantitative study without manipulation of treatments) are useful tools for answering questions related with nature; in our case, could lichens in the environment obtain their photobionts from the substrate in which they are growing? In fact, we found that the substrate underlying the lichens might be an additional source of available potential photobionts, since *Nostoc* dominated the cyanobacterial communities and many of the phylotypes retrieved matched the cyanobionts present in the *Peltigera* cyanolichens. Our findings provide a background for hypothesis testing and theory development for future studies of the lichenization process, encouraging analyses of photobiont availability to consider not only those that are symbiotically associated but also the potential photobionts that might be present in the substrates.

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