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Research Article

RESEARCH ARTICLE

Intrinsic factors of *Peltigera* lichens influence the structure of the associated soil bacterial microbiota

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One sentence summary: Lichen intrinsic and extrinsic factors shape the structure of the bacterial communities associated with lichens and the soil underneath them in a forested environmental context in Chilean Patagonia.
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ABSTRACT

Definition of lichens has evolved from bi(tri)partite associations to multi-species symbioses, where bacteria would play essential roles. Besides, although soil bacterial communities are known to be affected by edaphic factors, when lichens grow upon them these could become less preponderant. We hypothesized that the structure of both the lichen microbiota and the microbiota in the soil underneath lichens is shaped by lichen intrinsic and extrinsic factors. In this work, intrinsic factors corresponded to mycobiont and cyanobiont identities of *Peltigera* lichens, metabolite diversity and phenoloxidase activity and extrinsic factors involved the site of the forest where lichens grow. Likewise, the genetic and metabolic structure of the lichen and soil bacterial communities were analyzed by fingerprinting. Among the results, metabolite diversity was inversely related to the genetic structure of bacterial communities of lichens and soils, highlighting the far-reaching effect of these substances; while phenoloxidase activity was inversely related to the metabolic structure only of the lichen bacterial microbiota, presuming a more limited effect of the products of these enzymes. Soil bacterial microbiota was different depending on the site and, strikingly, according to the cyanobiont present in the lichen over them, which could indicate an influence of the photobiont metabolism on the availability of soil nutrients.

Keywords: terricolous cyanolichens; metabolite diversity; phenoloxidase activity; microbiota structure; *Nothofagus* forest; Chile

INTRODUCTION

Beyond the apparent self-sustaining status of lichens by its classical components (i.e. mycobiont and photobionts), and the general statement that they obtain their nutrients directly from the atmosphere and not from the substrate, these organisms might also get vital components with the help of other microbial members present on their thalli and in the surrounding environment (Grube *et al.* 2015; Sigurbjörnsdóttir, Andrésson and Vilhelmsson 2015; Spribille *et al.* 2016). In fact, difficulties on lichen culturing could be partly explained due to their nutritional dependence on the lichen-associated microbiota (Grube *et al.* 2015). However, the lichen microbiota could be playing not only roles in extra nutrient cycling, but also in defense/allelopathy against specific microbial groups such as pathogens (Hodkinson *et al.* 2012; Cernava *et al.* 2015). Accordingly, lichen symbioses have been proposed as resembling microecosystems (Grube *et al.* 2009; Hodkinson and Lutzoni 2009), comprising photobionts as producers, mycobionts as consumers and bacteria as contributors to nutrient acquisition, recycling and antagonism against other microbial groups (Aschenbrenner *et al.* 2016; Sigurbjörnsdóttir, Andrésson and Vilhelmsson 2016).

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Several authors have stated that bacterial communities associated with the lichen thallus are not a mere extension of those found in the substrate where the lichens grow, but can be considered as an additional component of the traditional symbiosis, mainly due to the similarity in abundance and the presence of specific groups of bacteria throughout different lichens (Bates *et al.* 2011; Cardinale *et al.* 2012; Printzen *et al.* 2012). Even more, a new group of Rhizobiales (LAR1) has been found as an exclusive member of the lichen microbiota (Hodkinson and Lutzoni 2009).

Several factors have been identified as descriptors of the structure of the lichen bacterial microbiota; among them, lichen intrinsic factors such as mycobiont identity (Grube and Berg 2009; Bates *et al.* 2011) and photobiont type (Hodkinson *et al.* 2012) and extrinsic factors such as biogeography and environmental context (Cardinale *et al.* 2008, 2012; Hodkinson *et al.* 2012; Printzen *et al.* 2012; Ramírez-Fernández *et al.* 2014). Nevertheless, the influence of these factors on the microbiota present in the substrate beneath the lichens has rarely been assessed, being roughly restricted to reports about rock or soil biological crusts (Bjelland *et al.* 2011; Castillo-Monroy *et al.* 2011; Bastida *et al.* 2014; Maier *et al.* 2014). These studies become important when considering that lichens act as generators of ecological niches for bacterial communities, either within the thalli or in the substrate below them (Bjelland *et al.* 2011).

Therefore, we hypothesized that both intrinsic and extrinsic lichen factors shape the structure, not only of the lichen bacterial microbiota (hereinafter referred to as lichen microbiota) but also of the soil bacterial microbiota underneath the lichen thallus (hereinafter referred to as soil microbiota), the former being more affected by intrinsic factors and the latter by extrinsic ones. We tested this hypothesis by the use of fingerprinting techniques at genetic and metabolic levels on terricolous *Peltigera* lichens growing in two sites of a native second-growth forest of *Nothofagus pumilio* (Poepp. & Endl.) Krasser 1896 in Chilean Patagonia (Till-Bottraud, Fajardo and Rioux 2012).

Lichens of the genus *Peltigera* are present in all continents; in Chile, only bipartite *Peltigera* cyanolichens have been reported (Martínez *et al.* 2003; Quilhot *et al.* 2012; Ramírez-Fernández *et al.* 2013; Zúñiga *et al.* 2015). In the case of *N. pumilio* forests in Chilean Patagonia, it has been described that their conservation stage had an influence on the diversity of *Peltigera* lichens growing in them (i.e. mature and young forests had higher diversity than grasslands derived from logged forests) (Ramírez-Fernández *et al.* 2013), which in turn affected the lichen-associated microbiota (Ramírez-Fernández *et al.* 2014). Nevertheless, the influence of *Peltigera* intrinsic factors on the soil microbiota where the lichens grow has not been evaluated.

Lichens in general are great producers of secondary metabolites, mainly by polymalonate, shikimate and mevalonate pathways (Boustie and Grube 2005). These metabolites could have antibiotic and allelopathic roles (e.g. Manojlović *et al.* 2012; Lokajová, Bačkorová and Bačkor 2014; Cernava *et al.* 2015), so it is expected that they affect the structure of the bacterial communities around the lichen thallus (Molnár and Farkas 2010; Hodkinson *et al.* 2012). However, *Peltigera* lichens are poor producers of these metabolites, and it has been proposed that phenoloxidase activity (e.g. laccases and tyrosinases) could provide a functional compensation because of the high oxidative capacity of their products (Beckett, Zavarzina and Liers 2003; Lisov *et al.* 2012), possibly acting allelopatically against certain bacterial groups.

In this study, we propose to analyze the influence of lichen intrinsic factors (specifically symbiont identities, metabolite production and phenoloxidase activity) and lichen extrinsic factors (the site of the forest where the lichens grow) in shaping the structure of bacterial communities associated with *Peltigera* thallus (i.e. lichen and soil microbiota).

Table 1. Distribution of symbiont pairs and conformation of samples.

Site	Mycobiont	Cyanobiont	n	Sample code
Site 1	M5	C01	10	S1M5C01
		C14	1	S1M5C14
	M6	C01	12	S1M6C01
	M8	C10	2	S1M8C10
Site 2	M1	C03	1	S2M1C03
	M2	C03	1	S2M2C03
	M4	C03	2	S2M4C03
	M5	C01	11	S2M5C01
		C03	3	S2M5C03
	M6	C01	6	S2M6C01
	M8	C12	1	S2M8C12

M: Mycobiont haplotype, C: Cyanobiont haplotype, n: number of thalli with those M and C haplotypes, in that site.

MATERIALS AND METHODS

Study sites and sampling

A total of 50 *Peltigera*-thallus fragments (~15 cm^2) and their associated substrate (i.e. soil) (~15 cm^3) were collected from two plots (~2 ha each), 300 m away from each other in a fragmented second-growth forest of *N. pumilio* (Till-Bottraud, Fajardo and Rioux 2012) at the Coyhaique National Reserve (Aysén Region, Chile; 45°31′42.96″S, 72°1′51.95″W; hereinafter referred to as Coyhaique). These two sites are close to pine tree plantations and differ from each other regarding their surrounding areas, site 2 being nearer than site 1 to open spaces such as rocky hillsides and a mallín (a kind of wetland). Lichen samples included in this study correspond to a subset of those previously reported by Zúñiga *et al.* (2015), in which the symbiont identity of these and other *Peltigera* lichens was determined. All samples were collected at least 1 m from the next closest thallus in order to minimize resampling of the same genetic individual.

Operational taxonomic units

The mycobiont and cyanobiont operational taxonomic units (OTUs) were defined as those groups of sequences that were 100% identical (nucleotide identity), using a distance analysis in Mega 5.2 software (Tamura *et al.* 2011). OTUs for mycobionts (based on concatenated sequences of LSU-SSU rRNA genes) and cyanobionts (based on SSU rRNA gene sequences) correspond to those published in Zúñiga *et al.* (2015), where they were named by an M or a C, respectively, followed by a number considering their order in the phylogenetic analyses (Table 1).

In the following methodologies, except the pre-treatment and DNA extraction, samples were organized according to their origin and symbiont identities. As can be seen in Table 1, this arrangement gave 11 samples originated from pooling different individuals, resulting in some composed of more than 10 single thalli/soils while others were represented by a single thallus/soil portion. Although lack of homogeneity due to this unequal composite size could not be totally discarded, the criteria for combining the samples are considered the key descriptors of the diversity of the bacterial communities studied in this work. Thus, it is expected that these communities are sufficiently homogeneous to justify their combination.

Pre-treatment of the samples and DNA extraction

Soil superficially associated with the collected portion of each thallus was removed with a brush and a spatula. Then, 80–100 mg of each cleaned thallus portion was mechanically fractioned with a minigrinder, and DNA was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., CA, USA) according to the manufacturer's instructions. In addition, DNA from 100 to 150 mg of the soil sample removed from each lichen thallus was extracted using the same kit. Quality and integrity of the extracted DNA were visualized in 0.8% (w/v) agarose gels in TAE 1X buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) stained with GelRed (Biotium, CA, USA). All DNA samples were stored in TE buffer (Tris-HCl 10 mM and EDTA 1 mM [pH 8.0]) at –20°C until further analysis.

Determination of lichen metabolites

Metabolites of the 11 lichen samples were determined by using bidimensional thin-layer chromatography (2D-TLC), based on descriptions by Culberson (1972) and modified by Menlove (1974). Compounds were extracted 5 min on hexane, 1 mL loaded on pre-coated silica gel 60 F254 aluminum plates (Merck, Darmstadt, Germany), and resolved in the solvent system EHF (diethyl ether : hexane : formic acid; 300 : 100 : 3 v/v/v) and TA (toluene : acetic acid; 20 : 3 v/v) as first and second dimension, respectively (Tønsberg and Holtan-Hartwig 1983), allowing the plates to dry for 10 min between each run. Metabolites were revealed under daylight, fixed by spraying the plate with sulfuric acid (10%) and then heated in an oven for 5 min at 110°C (Miadlikowska and Lutzoni 2000). In order to normalize migrations, spot distances from the origin were measured on both dimensions, and relativized to a spot present in every sample which appeared on the same region in each plate and had the same color on visible light. Then, relative retention factors (rRf) for each spot were obtained. In addition, visible light color (orange, blue, gray or other) and sulfuric acid reaction (positive or negative) of each spot were tabulated using a numeric code. This information was used in order to calculate similarities between samples, based on a neighbor-joining grouping analysis in PAST software v3.11 (Hammer, Harper and Ryan 2001). Subsequently, each cluster from the grouping analysis was considered as a different spot group, and the number of spots (i.e. abundance) in every group was used for calculating the Shannon (H') index of each sample in order to obtain a value reflecting the metabolite profile of each sample.

Quantification of phenoloxidase activity

Phenoloxidase activity was measured by quantifying the product of 2'2-azino-bis-[3-etilbenzotiazol-6-sulfonic] acid (ABTS) oxidation at 436 nm in 96-well plate assays, adapting the methods described by Laufer *et al.* (2006) and Zavarzina and Zavarzin (2006). From each sample, 8 mg of lichen thalli were homogenized using a minigrinder, and suspended in 300 μL of sodium acetate buffer 25 mM (pH 6). The homogenate was centrifuged at 7000 $\times$ *g* 5 min, and the supernatant was stored in 20% glycerol at –20°C until its use.

The enzymatic assay was carried out in duplicate with 500 ng of total protein per sample (quantified by the Bradford method). For that, 200 μL of sodium acetate buffer was added and then incubated 10 min at 22°C. In parallel, abiotic controls were assayed by adding 50 μL of 5 mM sodium azide (final concentration 1 mM) and 150 μL of sodium acetate buffer. Then, 50 μL of 2.5 mM ABTS (Sigma-Aldrich, MO, USA) was added (final concentration 0.5 mM).

The green coloration was measured at 436 nm (ε_{436} = 29 mM^{-1} cm^{-1}) in an Epoch microplate reader (Biotek, VT, USA). A phenoloxidase activity unit (U) was defined as micromoles of ABTS enzymatically oxidized by minute (subtracting the abiotic control value).

Terminal restriction fragment length polymorphism

Genetic structure of bacterial communities was determined by obtaining terminal restriction fragment length polymorphisms (TRFLP). For that, from the isolated DNA of the fragments of lichen thalli and their corresponding soils, bacterial SSU rRNA gene was amplified with forward primer 895F (Hodkinson and Lutzoni 2009), which excludes plastids and cyanobacteria, and reverse primer rP2 (Weisburg *et al.* 1991) labeled with 6-FAM (6-carboxyfluorescein) at the 5′ end. PCR mixes were prepared using GoTaq® Green Master Mix (GoTaq DNA polymerase in 1X 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). Cycling conditions consisted in an initial denaturalization step at 94°C for 3 min; then 30 cycles of 94°C for 30 s, 42°C for 30 s and 72°C for 1 min 30 s; and finally an extension at 72°C for 10 min. Quality and size of the amplicons were determined electrophoretically as described above except that 1.2% (w/v) agarose gels were used.

Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, WI, USA), and were independently incubated with three different restriction enzymes: *Alu*I, *Bsu*RI and *Hha*I (Thermo Fisher Scientific, MA, USA) according to the manufacturer´s instructions. After an alcohol DNA precipitation, products were resuspended in TE buffer and fragments were analyzed by capillary electrophoresis on an automated Genetic Analyzer ABI3730XL (Applied Biosystems; Macrogen Inc., Seoul, Korea). Size, height and area of the terminal restriction fragments (TRFs) were determined by comparison with the GeneScan 1200 LIZ® size-standard in GeneMapper software v3.7 (Applied Biosystems, CA, USA).

Only fragments of 30 bp or longer were considered in the data analysis, in order to discard peaks corresponding to PCR primers. Patterns from different samples were normalized to homogenize the total fluorescence units by an iterative standardization procedure. Additionally, profiles were manually aligned to avoid erroneous identification of TRFs by the expected shift in fragment sizes due to electrophoresis. Finally, the relative abundance of each TRF, as percentage, was determined by calculating the ratio between the height of the peak and the normalized total peak height of each sample.

Community level physiological profiles

Metabolic structure of bacterial communities was determined by obtaining the community level physiological profiles (CLPP) using EcoPlate (BioLog®, CA, USA), consisting of 96-well microplates with 31 different carbon sources plus one blank control, in triplicate.

In order to generate microbial suspensions from lichens, 100 mg of thalli from each sample were homogenized in 15 mL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4) and shaken overnight at 150 rpm and 25°C. Similarly, microbial suspensions from substrates were obtained from 2 g of soil, which was agitated at 150 rpm and 25°C for 1 h in 20 mL of PBS buffer. Then, lichen and soil suspensions were filtrated,

100 μL inoculated in each well of the corresponding microplate and incubated at 25°C for 1 week in a humid chamber. Bacterial community consumption of the carbon sources reduces a tetrazolium dye, whose purple coloration was daily measured at 590 nm in an Epoch microplate reader (Biotek).

Therefore, data were processed by subtracting the absorbance value at time zero, to minimize the interference of the sample color (Insam and Goberna 2004), and the absorbance value from the control (water). In the data analysis, absorbance values of 0.1 or higher were considered positive according to the detection limit of the reader.

The average well color development was calculated on each plate during the incubation time of the plates, and data were adjusted using the modified Gompertz equation (Zwietering *et al.* 1990) using OriginPro software v8.07 (OriginLab Corporation, MA, USA), in order to determine the incubation time when all the communities were in the exponential phase of each curve.

Data analyses

Based on the genetic and metabolic fingerprinting data, the structure of the bacterial communities (i.e. lichen and soil microbiota) was calculated using the Shannon (H') index. On the other hand, the similarity between these bacterial communities, considering their genetic and metabolic structures, was determined by principal component analyses (PCA) based on the Bray–Curtis dissimilarity index. In addition, analysis of similarity (ANOSIM) and similarity percentages (SIMPER) were calculated between the lichen and soil bacterial communities, grouping them independently by site, mycobiont and cyanobiont identities. All these analyses were done using PAST software v 3.11 (Hammer, Harper and Ryan 2001).

Finally, linear regressions between metabolite diversity, phenoloxidase activity and the structures of the bacterial communities were performed. For this, metabolite diversity and phenoloxidase activity were used as independent variables, while indices of genetic (TRFLP) and metabolic (CLPP) structures were used as dependent variables. These analyses were done in Graphpad Prism software v5.01 (Graphpad Software Inc., CA, USA).

RESULTS

Identity of lichen symbionts

Classification of lichen symbionts by the analysis of their ribosomal markers showed that, in the 50 samples of the two forest sites, there was a total of six mycobiont (M1, M2, M4, M5, M6 and M8) and five cyanobiont (C01, C03, C10, C12 and C14) haplotypes, whose names correspond to those in Zúñiga *et al.* (2015). The distribution of symbiont pairs in the *Peltigera* thalli is shown in Table 1 (e.g. pair M5-C01 was found in 10 thalli in site 1 and 11 times in site 2). Among the mycobionts, M5 and M6 were the most abundant, with 25 (50%) and 18 (36%) occurrences, respectively. In the case of cyanobionts, C01 was clearly the most abundant with 39 occurrences, and was also the only one present at both forest sites.

Mycobiont identities, based on phylogenetic analysis, correspond to those already reported in Zúñiga *et al.* (2015). The closest species (or group of them) to each mycobiont haplotype was as follows: M1 was related to *P. ponojensis*, M2 to *P. extenuata*, M4 to *P. rufescens*, M5 to *P. canina* group (species *P. boreorufescens, P. canina, P. evansiana, P. fuscopraetextata, P. pallidorufescens and P. praetextata*), M6 to *P. frigida* and M8 to *P. hymenina* group (species *P. hymenina, P. occidentalis, P. pacifica, P. polydactylon, P. pulverulenta and P. scabrosella*).

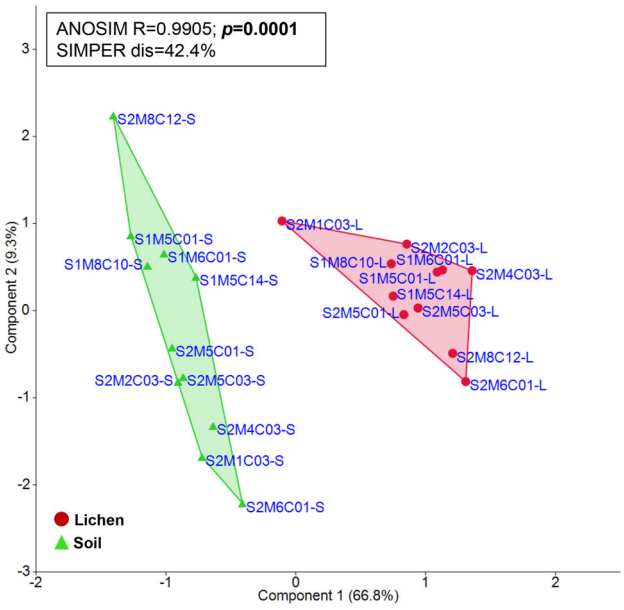

Figure 1. Multivariate PCA of the genetic structures of the bacterial communities. Profiles were obtained for each sample by TRFLP of the bacterial 16S rRNA genes, but excluding plastids and cyanobacteria, cut with three restriction enzymes. Lichen bacterial microbiota is indicated with red circles and soil bacterial microbiota with green triangles. In the box, the R and *p* values of ANOSIM and the dissimilarity value of SIMPER are indicated.

Lichen products

The lichen products assessed as potential descriptors of bacterial communities were metabolite diversity and phenoloxidase activity. Regarding the former, the color of the spots had previously been reported as a distinctive characteristic of lichen metabolites: orange for depsidones, blue for benzofurans and gray for fatty acids; meanwhile, sulfuric acid reaction reveals depsides and tridepsides (Culberson 1972).

Even though the spots on the 2D-TLC plates were lost after a short time of air dried, they could still be recorded and analyzed by a neighbor-joining grouping analysis, where eight different groups were obtained according to the similarities between the characteristics of the spots (i.e. rRf, color and sulfuric acid reaction). Then, considering the number of spots in these eight groups, the metabolite diversity in each sample was calculated. Statistically significant differences were not observed when comparing the metabolite diversity among different samples (Table S1, Supporting Information).

On the other hand, phenoloxidase activity of each composite sample showed values ranging from 9 to 113 Ug^{-1}, with a mean value of 60 Ug^{-1} of total protein. Among the 11 samples, phenoloxidase activity from sample S1M5C14 was significantly lower than the activity from S2M1C03 (Table S1).

Structure of bacterial communities

Genetic structures of lichen and soil microbiota were obtained for each sample by TRFLP with three different restriction enzymes. These profiles were analyzed by PCA (Fig. 1), where they were clearly separated in two groups, depending on the origin of the bacterial community (lichen or soil), with a SIMPER

Table 2. Genetic structure (TRFLP) data grouping analysis including results of ANOSIM (R and *p* values) and SIMPER (% of dissimilarity) multivariate analysis of samples grouped according to the site where they come from, and the mycobiont and the cyanobiont previously identified in the lichen thallus.

Origin	Grouped by	ANOSIM R	ANOSIM *p*	SIMPER dissimilarity (%)
Lichen	Site	–0.1349	0.8073	23.2
	Mycobiont	0.4628	**0.0421**	24.8
	Cyanobiont	0.1667	0.2073	24.5
Soil	Site	0.1984	0.0965	15.4
	Mycobiont	0.2340	0.1797	15.1
	Cyanobiont	0.3798	**0.0366**	15.5

Significant *p* values at the $p < 0.05$ level are in bold.

Table 3. Metabolic structure (CLPP) data grouping analysis including results of ANOSIM (R and *p* values) and SIMPER (% of dissimilarity) multivariate analysis of samples grouped according to the site where they come from, and the mycobiont and the cyanobiont previously identified in the lichen thallus.

Origin	Grouped by	ANOSIM R	ANOSIM *p*	SIMPER dissimilarity (%)
Lichen	Site	–0.0053	0.4212	62.6
	Mycobiont	0.6436	**0.0090**	68.3
	Cyanobiont	0.2868	0.1031	67.3
Soil	Site	0.7619	**0.0055**	69.2
	Mycobiont	–0.1809	0.7841	53.4
	Cyanobiont	0.4109	**0.0459**	58.4

Significant *p* values at the $p < 0.05$ level are in bold.

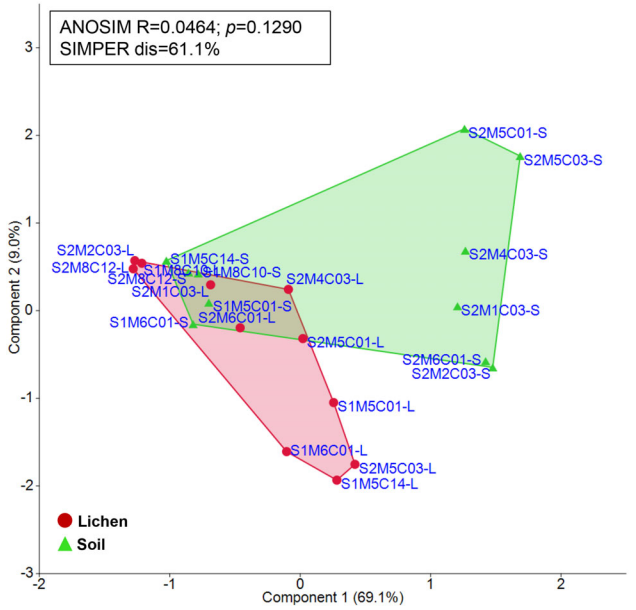

Figure 2. Multivariate PCA of the metabolic structures of the bacterial communities. Profiles were obtained for each sample by CLPP obtained using EcoPlate (BioLog). Lichen bacterial microbiota is indicated with red circles and soil bacterial microbiota with green triangles. In the box, the R and *p* values of ANOSIM and the dissimilarity value of SIMPER are indicated.

overall dissimilarity of 42.4%. ANOSIM confirmed this difference (R = 0.9905) with a statistically significant *p* value (0.0001). In order to analyze the association of location and symbiont identities with genetic bacterial structure, data were grouped by site, mycobiont and cyanobiont identity. In Table 2, ANOSIM and SIMPER analyses of the grouped TRFLP data are shown. The genetic structure of the microbiota of lichens was different depending on the mycobiont that was present in the thallus, whilst in the case of the genetic structure of the soil microbiota, the differences were observed depending on the cyanobiont present in the lichen.

Analogous to the genetic structure, metabolic profiles obtained using EcoPlate (BioLog), were analyzed by PCA (Fig. 2), and they did not show differences in an ANOSIM analysis (R = 0.0464) according to the origin of the bacterial community (lichen or soil); however, both groups showed a SIMPER overall dissimilarity of 61.1%. Then, data were grouped by site and symbiont identities, and results of ANOSIM and SIMPER analysis of the grouped CLPP data are shown in Table 3. The metabolic profiles showed differences when were grouped by mycobiont. On the other hand, differences were also observed when the metabolic structure of soil microbiota was grouped by site and by cyanobiont.

In order to analyze if levels of metabolite diversity and phenoloxidase activity were related to genetic and metabolic structures measured by the Shannon index, linear regression analyses were carried out. Metabolite diversity was inversely related to the genetic structure of both lichen (Fig. 3a) and soil microbiota (Fig. 3b). However, phenoloxidase activity levels were only inversely related to the metabolic structure, in this case just with the one associated with the lichen microbiota (Fig. 3g).

DISCUSSION

The lichen samples of this study (i.e. samples from Coyhaique) consisted in a subset of those from Zúñiga *et al.* (2015), which also included samples from other localities in Southern Chile and Antarctica. In particular, *Peltigera* lichens from Coyhaique represented 6 out of the total of 8 different mycobionts and 5 out of the total of 15 different cyanobionts reported in that work. In the case of the mycobionts, this high representativeness is consistent with the distribution patterns (Martínez *et al.* 2003) of at least the two most frequent mycobionts in both Coyhaique sites: M5 (*P. canina* group) and M6 (*P. frigida*), whose habitat has been described to be related to woodlands (Brodo, Scharnoff and Scharnoff 2001; Quilhot *et al.* 2012). Conversely, mycobionts M1, M2 and M4 were found exclusively in site 2, agreeing with reports of *P. ponojensis*, *P. extenuata* and *P. rufescens* mainly in open and disturbed sites (Brodo, Scharnoff and Scharnoff 2001). In contrast, regarding the cyanobionts, their low representativeness in Coyhaique might be explained by cyanobacterial endemism. In fact, cyanobiont C01 was clearly the most common in this study location (39 out of 50 thallus samples, 78%) and, aside from Coyhaique site, it was present only in a specimen from Navarino (one out of 60, 1.7%) (Zúñiga *et al.* 2015). It must be taken into account that the variation among lichen photobionts could be the result of photobiont-mediated guilds, which consist on a common pool of photobionts that could be forming a horizontally linked community of lichens growing in the same habitat (Rikkinen 2003; Dal Grande *et al.* 2014; Cornejo and Scheidegger 2016).

On the other hand, we studied the structure of bacterial communities associated with *Peltigera* lichens from Coyhaique (i.e. lichen and soil microbiota) through genetic and metabolic fingerprint-based approaches. Although some caveats and

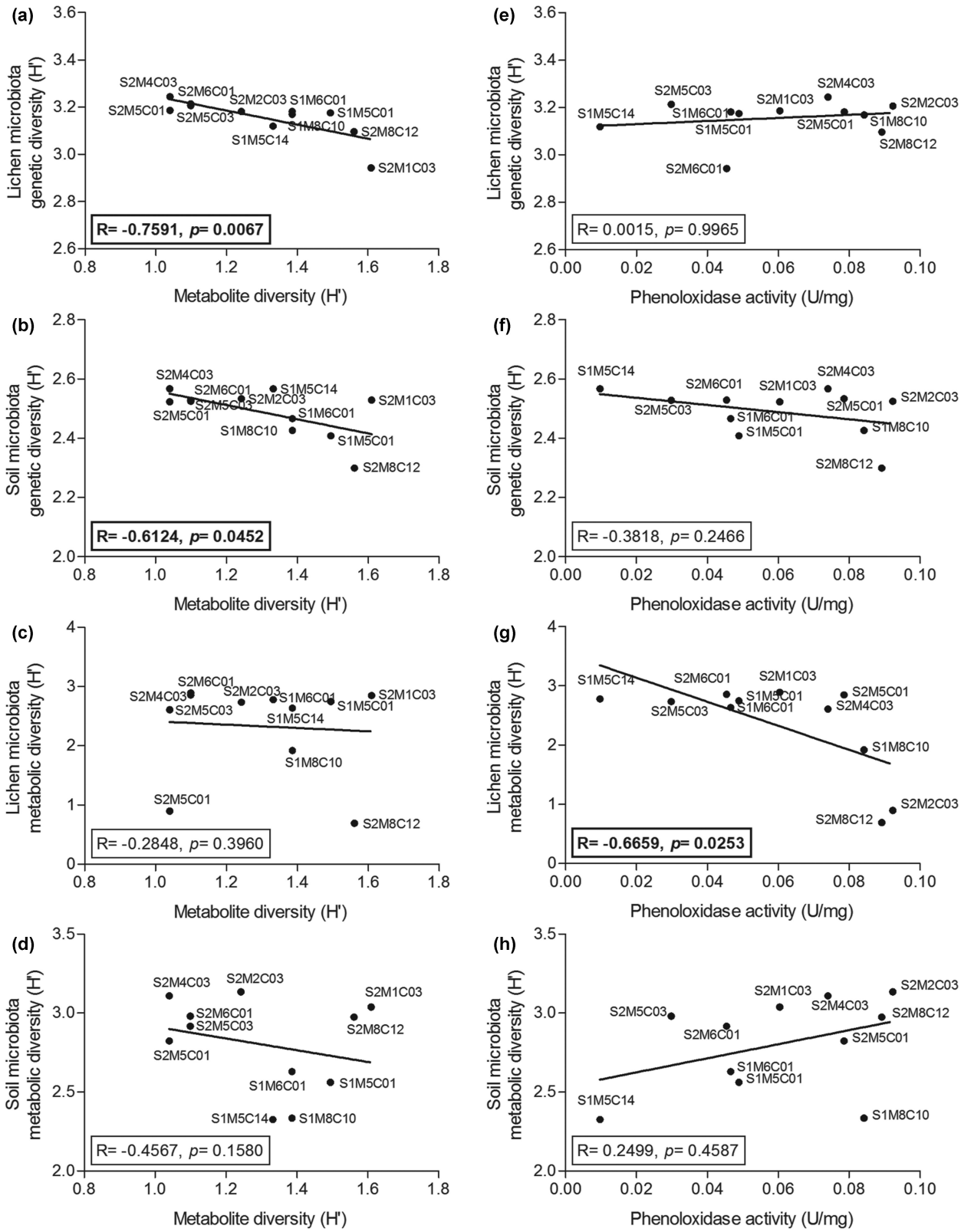

Figure 3. Linear regression analyses between lichen products and bacterial communities. The left panel shows the linear regression analyses between metabolite diversity and (a) genetic structure of lichen microbiota, (b) genetic structure of soil microbiota, (c) metabolic structure of lichen microbiota and (d) metabolic structure of soil microbiota. The right panel shows the linear regression analyses between phenoloxidase activity and (e) genetic structure of lichen microbiota, (f) genetic structure of soil microbiota, (g) metabolic structure of lichen microbiota and (h) metabolic structure of soil microbiota. Samples are plotted as points with the corresponding key name (S: site, M: mycobiont, C: cyanobiont). R and p values for each linear regression are indicated in the lower left corner of each graph area, and shown in bold letters when significant.

limitations such as those associated with DNA extraction (Martin-Laurent *et al.* 2001), PCR amplification (Wilson 1997), molecular marker copy numbers (Crosby and Criddle 2003) and culture-dependence in the case of BIOLOG® assays (Orlando *et al.* 2007) need to be addressed, fingerprinting is still a valuable tool to assess changes in microbial community diversity by allowing cost-effective analyses of several samples and, thus, to develop robust answers (van Dorst *et al.* 2014; Almasia *et al.* 2016). In this work, the use of the TRFLP technique with three different restriction enzymes allows variance estimation of closely related bacterial groups, reaching similar findings as other common methods such as DGGE (denaturing gradient gel electrophoresis) and SSCP (single-stranded conformational polymorphism) (Smalla *et al.* 2007). In addition, the CLPP assay, based on EcoPlate (BioLog), offers the potential to explore functional traits of microbial communities. To our knowledge, this carbon source utilization approach was only used to describe bacterial communities associated with lichens in the context of biological soil crusts, which are known to harbor more complex communities than a single lichen thallus (Yu *et al.* 2012; Bastida *et al.* 2014).

The microbiota of lichens was clearly distinct from the microbiota of soils in their genetic structure, proving the hypothesis that lichen microbiota is not a mere extension of the microbial communities present in the substrate where lichens grow. In this regard, several studies have shown that the identity of the symbionts influences the diversity of the microbial communities closely associated with lichens (Grube and Berg 2009; Bates *et al.* 2011; Hodkinson *et al.* 2012), but they rarely studied which specific lichen intrinsic factors could be causing that impact.

In order to assess in what way the lichen microenvironment affects the bacterial community structure, we measured two well-known lichen factors: metabolite production (Molnár and Farkas 2010) and phenoloxidase activity (Beckett, Zavarzina and Liers 2013). As expected, the microbiota of lichens had different structure depending on the mycobiont identity at both genetic and metabolic levels. In turn, inverse relations were found between the diversity of lichen metabolites and the bacterial genetic structure, and also between the phenoloxidase activity and the bacterial metabolic structure. These different effects could be explained by the specific action of metabolites (Karazog *et al.* 2009; Mitrović *et al.* 2011) versus the generalist impact of oxidative products on sensitive bacterial populations (Lisov *et al.* 2012).

In the case of the soil microbiota, an environmental significant influence, i.e. that caused by lichen extrinsic factors attributable to the site of the forest where the lichens grow, was observed at the metabolic structure level. These differences could be explained by the influence of surrounding areas or by diversity patterns of *Peltigera* species found in both sites, which would involve other intrinsic factors to those studied here. To the extent of our knowledge, the influence of this kind of factors on the lichen-associated soil microbiota has been previously assessed only considering the conservation state of forests in Tierra del Fuego Island (Ramírez-Fernández *et al.* 2014). However, an environmental effect on the structure of the lichen microbiota at a genetic level, due to geographical location, has been observed before in lichen communities but from very distant sites (up to 7700 Km) (Cardinale *et al.* 2012; Hodkinson *et al.* 2012). Strikingly, lichen intrinsic factors, i.e. those affecting the lichen microenvironment, showed an influence also on the structure of soil microbiota, at both metabolic and genetic levels. One of them was the metabolite production and another one was the cyanobiont identity.

The effect of metabolite diversity, not only on the lichen microbiota but also on the soil microbiota (Akpinar, Ozturk and Sinirtas 2009), evidences the far-reaching influence of these lichen products. In fact, metabolite effects over bacterial communities have been shown even using a single molecule at physiological concentration levels in an experimental assay tested by TRFLP (Persson *et al.* 2011). Conversely, phenoloxidase activity was not related to the structure of soil microbiota, indicating its more generalist impact just only in the vicinity of the lichen thallus, which could be related rather to a metabolic resistance of the bacterial communities (Baldrian 2004), probably due to the biological ubiquity of the activities of these enzymes.

On the other hand, the cyanobiont identity effect over soil communities is an unexpected result considering that lichens are mainly conformed by fungal biomass. Photobiont influence on bacterial communities closely related to lichen thalli has been described before by Hodkinson *et al.* (2012), but just according to the photobiont type (i.e. cyanobacterial or green-algal symbiont). These authors discuss that photosynthetic rates and nitrogen-fixing capabilities of the photobionts could result in different available concentrations of carbon and nitrogen for the holobiont, which in turn can lead to a bacterial community differentiation. An alternative explanation could be that lichen substrates might be enriched in cyanobionts from the lichens, since lichen thalli are a recognized source of photobionts in horizontal transmission or in photobiont switching (Rikkinen 2003; Muggia *et al.* 2013); however, further evaluation of the availability of potential cyanobionts in the substrate microbiota is necessary. In this context, Delgado-Baquerizo *et al.* (2015) found a positive relation between nutrient concentration (carbon, nitrogen and phosphorus) in biocrust-forming lichen thalli and the substrate below them. It seems reasonable then to propose that differences in carbon and nitrogen-fixing rates between photobionts could generate different nutrient availabilities to microbial communities in the soil below lichens (Knowles, Pastor and Biesboer 2006), and while in our work only *Nostoc* cyanobionts were analyzed, intrageneric metabolic differences could occur. Although soil factors (e.g. water content, pH and cation exchange capacity) certainly impact soil microbial composition (Docherty *et al.* 2015), these factors could become less important in the case of soils influenced by lichens (Delgado-Baquerizo *et al.* 2015). In fact, it is possible that lichens recruit their associated microbiota from soil similarly as plants do through their rhizosphere, creating particular environmental conditions for the survival of specific bacterial groups (Philippot *et al.* 2013).

In conclusion, lichen products can structure even the soil bacterial communities present below them, highlighting their role as possible drivers of natural microenvironmental conditions and evidencing the need of further analyses about how particular soil microbial communities are being shaped by lichens upon them.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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