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"MEASURING THE FACTORS INVOLVED IN THE SUCCESSFUL ESTABLISHMENT OF THE CYANOLICHEN PELTIGERA"

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Por

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FACULTAD DE CIENCIAS

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INFORME DE APROBACION

TESIS DE DOCTORADO

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata:

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A MI SONY VAIO QUE DIO SU VIDA POR ESTA TESIS...

RESUMEN BIOGRÁFICO

Nací en la ciudad de Santiago el 19 de Junio de 1983 y realicé toda mi enseñanza escolar en el colegio Lo Castillo, del cual egresé en el año 2000. En el año 2001 ingresé a la carrera de Ingeniería en Biotecnología Molecular de la Facultad de Ciencias, Universidad de Chile, egresando como Licenciada en el año 2006. Mi tesis de pregrado la desarrollé en el laboratorio de Ecología Microbiana bajo la tutoría de la Dra. Margarita Carú, titulándome de Ingeniero en Biotecnología Molecular en el año 2008.

En el año 2010 ingresé al programa de Doctorado en Ciencias con mención en Microbiología, aprobando mi Examen de Calificación y Proyecto de Tesis en Agosto del año 2012, bajo la tutoría de la doctora Julieta Orlando. Finalmente defendí y aprobé mi examen privado de tesis en Octubre del 2014 y el examen público en Enero del 2015, obteniendo mi grado de Doctor en Ciencias.

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Querida Catita

Lo has hecho una vez más; cuando habíamos pensado que habías tocado el cielo y por fin te establecías con confianza y seguridad y te posesionabas de tu maravillosa profesión, surgió de pronto un nuevo eslabón en esta larga carrera investigativa, para dar paso a nuevos tiempos y mayores conocimientos.

Sí, lo has hecho una vez más, porque tal vez pertenezcas a un grupo de privilegio y excelencia en el campo de las ciencias, o porque tuviste en un momento la suerte y la oportunidad de seguir creciendo y desarrollándote profesionalmente, o quizás porque en la actualidad el mundo científico y tecnológico se desenvuelve y crece en forma vertiginosa, haciéndose insuficiente todo lo que poseías hasta ese instante. Lo cierto hija mía, es que una vez más, y a punto de superar esta nueva valla, nos sorprendes con tus capacidades, talentos y características que sobrepasan la normalidad.

Esta nueva conquista va de la mano con la consecución de nuevos horizontes, y tal vez ya te prepares para ir mucho más lejos en busca de nuevos objetivos. Creo, desde mi humilde perspectiva, que recién ahora estás iniciando un camino, el cual se extenderá quizás toda la vida haciéndola fascinante y encantadora, atractiva y cautivante.

Ya no serán tus padres quienes te guiarán por los senderos de la existencia, ni siquiera tú eres dueña de tu destino, todo lo que se presente en adelante irá siempre precedido de opciones, ofertas, elecciones y alternativas. Estás en medio del torbellino de la vida y sólo te queda a tu haber contar con un poco de suerte y saber elegir lo que te convenga. A pesar de todo, creo que estás bien encaminada y, por la formación que posees, tus respuestas a los inconvenientes y tropiezos serán siempre acertadas.

Sigue prosperando y, en cuestión de emociones y de afecto, cuenta con tus padres. El resto lo resolverás tú, tienes capacidades para ello.

Suerte

Te quiere, papá

Diciembre 2014

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LIST OF ABBREVIATIONS

AICc:	Corrected Akaike Information Criterion
Av Se_i:	Average Selectivity
BI:	Bayesian Inference
bp:	Base Pairs
C:	Coyhaique
Cs:	Cyanobionts
(a)Cs:	Total Available Potential Cyanobionts
(l)Cs:	Lichenized Cyanobionts
(s)Cs:	Available Potential Cyanobionts in the Substrate
CF1:	Coyhaique Forest 1
CF2:	Coyhaique Forest 2
CI:	Confidence Interval
D:	Deception
dNTP:	Deoxyribonucleotide triphosphate
DH:	Deception Hillside
E_i^*:	Vanderploeg & Scabia's Electivity Index
ITS:	Internal Transcribed Spacer
ITS-HR:	ITS Hyper-Variable Region
K:	Karukinka

KG:	Karukinka Grassland
KM:	Karukinka Mature Forest
KY:	Karukinka Young Forest
LBG	Latitudinal Biodiversity Gradient
LFF:	Lichen-forming Fungi
LPSp:	Local Phylogenetic Specificity
LPSpR:	Local Phylogenetic Specificity Rank
LSU rDNA:	Large Sub-unit of the Ribosomal DNA
ML:	Maximum Likelihood
Ms:	Mycobionts
N:	Navarino
NG:	Navarino Grassland
NCBI:	National Center for Biotechnology Information
NM:	Navarino Mature Forest
NY:	Navarino Young Forest
OTU:	Operational Taxonomic Unit
Se_i:	Selectivity Index
Se_iR:	Selectivity Index Rank
SSU rDNA:	Small sub-unit of the Ribosomal DNA
WSD:	Weighted Shannon Diversity
WSDR:	Weighted Shannon Diversity Rank

ABSTRACT

Lichens are stable symbiotic associations formed between a fungus (the *mycobiont*) and a photosynthetic microorganism (the *photobiont*) which can be a green alga and/or a cyanobacterium. In this last case, they are termed *cyanolichens* and are of particular interest due to their nitrogen fixing capacity.

The ecological and genetic factors involved in the development of a successful lichen symbiosis are still poorly understood. A conceptual model of lichenization suggests that *photobiont availability* in a given locality is the first factor determining which lichens can occur, and this factor depends mainly on the photobiont's dispersal capacity and the ecological conditions of the place. If there is not a suitable photobiont available, most lichen fungi will not survive in the free-living state. Second, from the perspective of the mycobiont, the successful establishment of the symbiosis requires a specific genetic compatibility with the available photobionts, factor known as *specificity* and defined as the taxonomic range of the photobionts with which a given mycobiont can be associated. Third, even compatible pairings in one habitat may not be optimal in another, leaving only a portion of the possible associations detectable at any site. This factor is known as *selectivity* and corresponds to the preferential association of a mycobiont with a subset of its available specific partners. It is not clear what drives selectivity, but it has been suggested to depend on geographical and ecological factors. Lastly, the environmental fitness of the associations, or *ecological success*, is directly related to the local

abundance of the different symbiotic pairs, reflecting a combination of the above-mentioned factors.

Most of the current research on biodiversity and conservation of lichens states that changes in forest cover have an effect on the diversity of both, epiphytic and terricolous species. In the specific case of *Peltigera*, diversity has been reported to be higher in undisturbed native forests of *Nothofagus* spp. than in degraded un-forested environments such as grasslands, both in Tierra del Fuego, Chile. On the other hand, studies also suggest that diversity decreases at higher latitudes, concept known as *latitudinal biodiversity gradient* (*LBG*) and rarely studied for lichen associations. Given that selectivity has been suggested to decrease in more adverse environments, in this work it was proposed that mycobionts within the genus *Peltigera* would be more selective for their specific cyanobionts in forested matrices than in un-forested ones and, in the case of the former, would be more selective at lower latitudes. The study model corresponded to the cyanolichen *Peltigera* and samples were collected from 4 sites that included 9 habitats in Southern Chile and Antarctica, which are very diverse but still poorly studied in lichenological terms.

In order to establish the operational taxonomic units (OTUs) of the symbionts, the genetic diversity of the LSU and SSU rDNA molecular markers was assessed for the mycobionts and cyanobionts, respectively. Among the 186 samples collected, 8 mycobiont and 15 cyanobiont OTUs were defined according to a 100% nucleotide identity criterion. Phylogenetic analyses comparing these OTUs with sequences obtained from key works in the phylogeny of *Peltigera* and *Nostoc* showed that they all belonged to sections *Peltigera, Horizontales* and *Polydactylon*, in the case of the mycobionts, and

to the *Nostoc* clade II and *Peltigera* guild, in the case of the cyanobionts. Based on these results, we suggest that *Peltigera* diversity described to date in the studied regions using traditional morphological surveys has underestimated the true diversity present, and we recommend further exploration of these areas.

The diversity of the symbionts was calculated using the weighted Shannon diversity index, which showed that the mycobionts were more diverse in the forested matrices than in the un-forested ones, while the cyanobionts were similarly diverse in both types of matrices. In addition, the mycobionts showed a diversity gradient inversely correlated with the latitude of the sampling sites, while the cyanobionts did not show this relation.

The availability of the cyanobionts, on the other hand, was defined as the sum of the non-lichenized potential cyanobionts detected directly from the substrates where the lichens grow through the construction of clone libraries, plus the lichenized ones present in the same sites. In most cases, cyanobionts were more abundant in their lichenized state than non-lichenized in the substrates and all the non-lichenized potential cyanobionts detected in the substrates corresponded to those that were also lichenized in a given habitat.

Specificity, on the other hand, was calculated by adapting the concept of *phylogenetic specificity* originally designed for host-parasite interactions but never used for studying lichen associations. The most specific mycobionts from this work belonged to sections *Peltigera* (M3) and *Horizontales* (M7); however, the spectrum was broader within the first, having also mycobionts with intermediate and low values, M1 being the least specific mycobiont. Even though some of the cyanobionts were shared between the mycobionts, some *Peltigera* specimens housed their own specific *Nostoc* strains without

sharing them with each other, as was the case of mycobiont M8 and the above-mentioned M3, whose only cyanobiont, C8, was exclusively associated with this mycobiont (*one to one* association). Moreover, neither M3 nor C8 sequences were strongly related with any *Peltigera* or *Nostoc* from the database, suggesting that this association might correspond to a new, yet un-described species.

Finally, in order to calculate the selectivity of the mycobionts, an *electivity index* initially developed for evaluating the utilization by animals of different types of food was adapted for the lichen association. This *selectivity index* (Se_i) considered between its variables the availability of the cyanobionts, the specificity of the mycobionts and the ecological success of the symbiotic pairs. To date there are no indices for quantitatively determining selectivity in lichen associations, which makes the present one a valuable tool for comparative purposes.

When determining the selectivity of each mycobiont, results showed that the least selective ones (M4, M5 and M8) were the most ecologically successful and, in addition, were related to *Peltigera* species with a wide global distribution range (*P. rufescens*, *P. canina* lineage and *P. hymenina* lineage). On the contrary, the most selective ones (M1, M2 and M6) were the least successful and were related to species with narrower global distributions (*P. ponojensis*, *P. extenuata* and *P. frigida*), suggesting that higher selectivities might decrease the capacity of lichens to colonize broader areas.

As a final point, there was no relation between mycobiont selectivities and the type of matrix in which the lichens were growing (forested or un-forested), and there was no tendency relating their selectivity with the latitude increase of the sampling sites.

RESUMEN

Los líquenes son asociaciones simbióticas estables que se establecen entre un hongo (el *micobionte*) y un microorganismo fotosintético (el *fotobionte*), el cual puede ser un alga verde o una cianobacteria. En este último caso se denominan cianolíquenes y son de particular interés debido a su capacidad de fijación de nitrógeno.

Los factores ecológicos y genéticos que determinan el desarrollo de una simbiosis exitosa son aún pobremente entendidos. Un modelo conceptual de liquenización sugiere que la *disponibilidad de los fotobiontes* en una localidad dada es el primer factor que determinaría qué líquenes se encuentran presentes, y este factor depende principalmente en la capacidad de dispersión del fotobionte y de las condiciones ecológicas del lugar. Si no existe un fotobionte adecuado disponible, la mayoría de los hongos formadores de líquenes no sobrevivirán en estado de vida libre. Segundo, desde la perspectiva del micobionte, el establecimiento exitoso de la simbiosis requiere de una compatibilidad genética específica con los fotobiontes disponibles, factor conocido como *especificidad* y definido como el rango taxonómico de los fotobiontes con los cuales un dado micobionte puede estar asociado. Tercero, aún parejas compatibles en un hábitat pueden no ser óptimas en otro, dejando sólo una porción de las posibles asociaciones detectable en algún lugar. Este factor es conocido como *selectividad* y corresponde a la asociación preferencial de un micobionte con un sub-grupo de sus compañeros específicos disponibles. No está claro qué determina la selectividad, pero se sugiere que dependería de factores geográficos y ecológicos. Por último, la aptitud ambiental de las

asociaciones, o *éxito ecológico*, está directamente relacionada con la abundancia local de los diferentes pares simbióticos, reflejando una combinación de los factores mencionados anteriormente.

La mayoría de la investigación actual en temas de biodiversidad y conservación de líquenes establece que cambios en la cobertura boscosa tienen un efecto en la diversidad de especies tanto epífitas como terrestres. En el caso específico de *Peltigera*, se ha reportado mayor diversidad en bosques nativos de *Nothofagus* spp. que en ambientes degradados como praderas, ambos en Tierra del Fuego, Chile. Por otro lado, estudios también postulan que la diversidad decrece a mayores latitudes, concepto conocido como *gradiente latitudinal de biodiversidad* (*LBG*) y raramente estudiado para asociaciones liquénicas. Dado que se ha sugerido que la selectividad decrece en ambientes más adversos, en este trabajo se propuso que micobiontes del género *Peltigera* serían más selectivos por sus cianobiontes específicos en matrices boscosas que en no boscosas y que, en el caso de las primeras, serían más selectivos a menores latitudes. El modelo de estudio correspondió al cianoliquen *Peltigera* y las muestras se recolectaron desde 4 sitios que incluyeron 9 hábitats en el sur de Chile y la Antártica, los cuales son muy diversos pero aún pobremente estudiados en términos liquenológicos.

Con el fin de establecer las unidades taxonómicas operacionales (OTUs) de los simbiontes, la diversidad genética de los marcadores moleculares LSU y SSU rDNA fue determinada para los micobiontes y cianobiontes, respectivamente. Dentro de las 186 muestras colectadas, se definieron 8 OTUs de micobiontes y 15 de cianobiontes de acuerdo a un criterio de 100% de identidad nucleotídica. Análisis filogenéticos comparando estas OTUs con secuencias obtenidas desde trabajos clave en la filogenia de

Peltigera y *Nostoc* mostraron que todas pertenecían a las secciones *Peltigera*, *Horizontales* y *Polydactylon*, en el caso de los micobiontes, y al clado *Nostoc* II y al gremio *Peltigera*, en el caso de los cianobiontes. Con base en estos resultados, sugerimos que la diversidad de *Peltigera* descrita a la fecha en las regiones estudiadas, usando clasificaciones morfológicas tradicionales, ha subestimado la verdadera diversidad presente y recomendamos una mayor exploración de estas áreas.

La diversidad de los simbiontes se calculó usando el índice de Shannon ponderado, el cual mostró que los micobiontes eran más diversos en las matrices boscosas que en las no boscosas, mientras que los cianobiontes eran similarmente diversos en ambos tipos de matrices. Además, los micobiontes mostraron un gradiente de diversidad inversamente correlacionado con la latitud de los sitios de muestreo, mientras que los cianobiontes no mostraron esta relación.

La disponibilidad de cianobiontes, por otro lado, se definió como la suma de los potenciales cianobiontes no liquenizados detectados directamente desde los sustratos donde crecían los líquenes a través de la construcción de librerías de clones, más aquellos cianobiontes liquenizados presentes en los mismos sitios. En la mayoría de los casos, los cianobiontes fueron más abundantes en su estado liquenizado que no liquenizado en los sustratos y todos los potenciales cianobiontes no liquenizados detectados en los sustratos correspondieron a aquéllos que se encontraban también liquenizados en un dado hábitat.

La especificidad, por otra parte, se calculó adaptando el concepto de *especificidad filogenética* originalmente diseñado para interacciones hospedero-parásito, pero nunca antes usado para el estudio de asociaciones liquénicas. Los micobiontes más específicos

de este trabajo pertenecieron a las secciones *Peltigera* (M3) y *Horizontales* (M7); sin embargo, el espectro fue más amplio dentro de la primera, presentando también micobiontes con valores bajos e intermedios, siendo M1 el micobionte menos específico. A pesar de que algunos cianobiontes fueron compartidos entre los micobiontes, ciertos especímenes de *Peltigera* presentaron sus propias cepas específicas de *Nostoc*, sin compartirlas entre ellos, como fue el caso del micobionte M8 y el ya mencionado M3, cuyo único cianobionte, C8, estuvo exclusivamente asociado con este micobionte (asociación *uno es a uno*). Más aún, ni las secuencias de M3 ni de C8 se asociaron robustamente con alguna de *Peltigera* o *Nostoc* de la base de datos, sugiriendo que esta asociación podría corresponder a una nueva especie aún no descrita.

Finalmente, con el fin de calcular la selectividad de los micobiontes, un *índice de elegibilidad* inicialmente desarrollado para evaluar la utilización por parte de animales de diferentes tipos de comida se adaptó para la asociación liquénica. Este *índice de selectividad* (Se_i) consideró entre sus variables la disponibilidad de los cianobiontes, la especificidad de los micobiontes y el éxito ecológico de los pares simbióticos. A la fecha, no existen índices para determinar cuantitativamente la selectividad en asociaciones liquénicas, lo cual hace del presente índice una valiosa herramienta para propósitos comparativos.

Al determinar la selectividad de cada micobionte, los resultados mostraron que los menos selectivos (M4, M5 y M8) fueron los más ecológicamente exitosos y, además, se relacionaron a especies de *Peltigera* con un amplio rango de distribución global (*P. rufescens,* linaje *P. canina* y linaje *P. hymenina*). Por el contrario, los más selectivos (M1, M2 y M6) fueron los menos exitosos y se relacionaron a especies con una

distribución global más estrecha (*P. ponojensis*, *P. extenuata* y *P. frigida*), sugiriendo que selectividades más altas podrían disminuir la capacidad de los líquenes de colonizar áreas más amplias.

Como último punto, no hubo relación entre la selectividad de los micobiontes y el tipo de matriz en que los líquenes estaban creciendo (boscosa o no boscosa), y tampoco se observó alguna tendencia que relacionara su selectividad con el aumento de latitud de los sitios de muestreo.

INTRODUCTION

1.1. Lichen symbiosis

Lichens are a symbiotic association of a fungus and a photosynthetic microorganism (green alga and/or cyanobacterium) resulting in a stable body or thallus of specific structure (Ahmadjian, 1982a), the *holobiont*, that does not resemble either symbiont in the free-living (=unlichenized) state (Ahmadjian, 1982b). In symbiosis the fungus is termed *mycobiont* and the photosynthetic partner is named *photobiont*.

Lichens can be bi- or tripartite organisms, with bipartite ones being the most common and consisting of a fungus and a photosynthetic alga or a cyanobacterium. On the other hand, tripartite lichens occur in 3-4% of lichens and have an alga as their primary photobiont, with a cyanobacterium as a secondary photobiont generally located in specialized structures termed cephalodia and mainly dedicated to N_2 fixation (Nash, 2008; Fedrowitz, 2011). All lichens whose photobiont is a cyanobacterium are called *cyanolichens* and comprise around 10 to 15% of the currently known lichen symbioses (Rikkinen *et al.*, 2002; Nash, 2008). The most frequent cyanobacterial genus in cyanolichens is *Nostoc*, a photosynthesizing and N_2-fixing cyanobacterial genus which also participates in a wide range of symbiotic associations with hosts from different taxonomic groups such as bryophytes, pteridophytes, gymnosperms and angiosperms (Rai *et al.*, 2002). Even though it is clear that the other partner gains either photosynthates or combined nitrogen from associating with *Nostoc* (depending on the type of host), the consequences for *Nostoc* to participate in these associations are not as

obvious (Paulsrud, 2001). For that reason some lichen forming fungi (LFF) have been regarded as controlled parasites of the photobionts, given that they live in a biological equilibrium with the photosynthetic organism (Ahmadjian & Jacobs, 1981). However, the term mutualism is probably a more suitable descriptor of the lichen symbiosis since the photobiont host usually suffers no appreciable harm and actually receives some benefits like a shield from excessive sunlight, desiccation and mechanical injury, along with inorganic compounds from its fungal host (Easton, 1994).

Systematically, lichens are a non-monophyletic group of fungi with about 98% of the species belonging to Ascomycota and a few lichenized Basidiomycetes and Deuteromycetes (= Fungi Imperfecti) (Nash, 2008). With respect to their taxonomic classification, this is exclusively based on the fungus since it is the component playing the primary role in determining the lichen's form (Nash, 2008).

Approximately one fifth of all known fungi have been described as obligated lichen forming species (Kirk *et al.*, 2001), reflecting the evolutionary success of this symbiotic association. In fact, the success of this life strategy becomes very evident since lichens can be found in almost all terrestrial ecosystems with a few also occurring in freshwater or even submerged in marine environments (Galloway, 2008). Another measure of their ecological success is their nearly ubiquitous ability to colonize different substrates. They can grow on or inside rocks, on or inside woody-plant barks, on wood, soil, mosses, leaves of vascular plants, on other lichens and on man-made substrates such as concrete, glass, metals and plastics (Lutzoni & Miadlikowska, 2009). Even more, since the diversity of lichens has been determined mainly based on morphological characters, it is likely that the total number of LFF is still underestimated due to the existence of cryptic

species, whose morphological characters are either not evident macroscopically or have been generally overlooked, being only distinguished by molecular analyses (Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011).

On the basis of their overall habit, lichens are traditionally divided into three main morphological groups: crustose, foliose and fruticose types. Crustose lichens are tightly attached to the substrate with their lower surface and may not be removed from it without destruction. Water loss is restricted primarily to the upper exposed surface and, when growing on inclined rock surfaces, they profit from surface water flow. These features allow these organisms to tolerate extreme habitats such as bare, exposed rock surfaces. Foliose lichens, on the other hand, are leaf-like, flat and only partially attached to the substrate. Typically they have a dorsi-ventral organization with distinct upper and lower surfaces. They develop a great range of thallus sizes and diversity, which is often divided into lobes showing various degrees of branching. Finally, fruticose lichens present hair-like thallus lobes, which can be strap-shaped or shrubby and the lobes may be flat or cylindrical. They always stand out from the surface of the substrate and the majority possesses radial symmetric thalli (Büdel & Scheidegger, 2008).

1.2. Reproduction in lichens

Lichens have developed a broad range of reproductive strategies, which include either sexual or asexual reproduction of the fungus (re-lichenization and symbiotic reproduction of the lichen, respectively). Sexual spores are in most cases aposymbiotic, i.e. are separated from the symbiotic state, and as a consequence, re-lichenization has to take place at the beginning of each life cycle because the development of the

characteristic lichen phenotype and the development of reproductive structures are dependent on the symbiotic state. However, various lineages of lichen-forming fungi reproduce asexually and have developed a broad range of vegetative, symbiotic propagules (Honegger, 2008).

Since nearly all LFF have never been reported growing without a photoautotroph they are considered to be obligate symbionts and, for that reason, the mycobiont is seen as the most dependent of the two symbiotic partners (Lutzoni & Miadlikowska, 2009). The general life cycle of lichens is summarized in Figure 1.

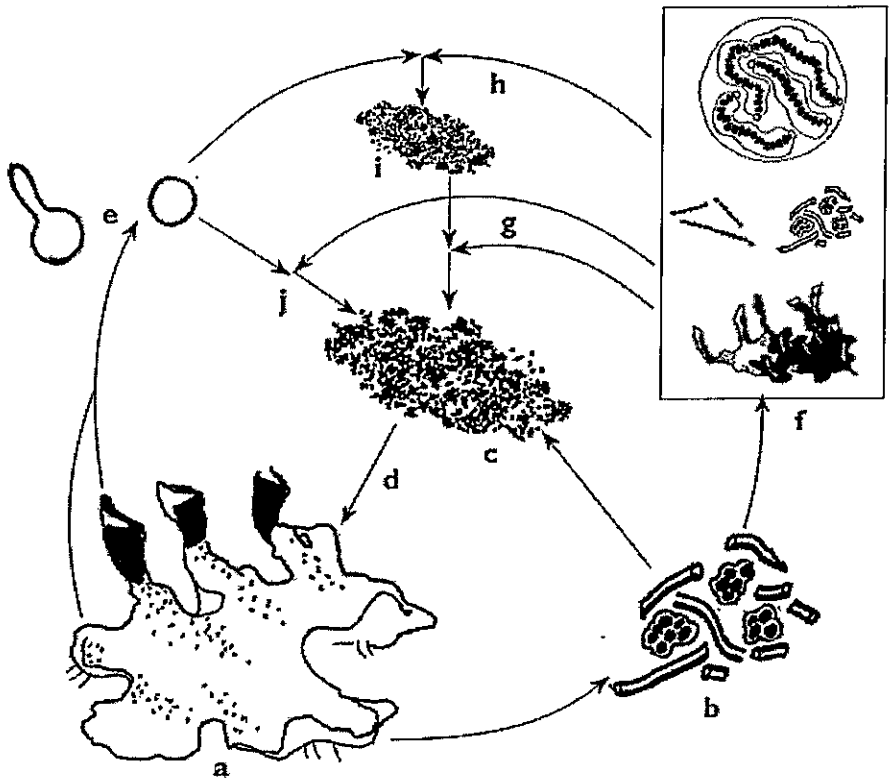


Figure 1. Schematic representation of reproductive strategies in lichens (see explanation in the text). (a) Mature thallus, (b) Vegetative propagules, (c) Germination of the propagules, (d) Pre-thallus differentiation, (e) Fungal release of spores, (f) Photobiont sources, (g) Compatible photobionts, (h) Incompatible photobionts, (i) Pre-thallus, (j) Fungal spore association with compatible photobiont.(Paulsrud, 2001).

The mature thallus (a) may contain structures specialized for the dispersal of the mycobiont alone as well as together with its photobiont. When the fungus is dispersed as vegetative propagules of photobiont and mycobiont together (b), such as thallus fragments or specialized symbiotic diaspores (soredia, isidia), it can readily establish a new lichen. In this case, first an undifferentiated pre-thallus (c) is formed, followed by the formation of a more organized and stratified thallus (d). Both asexual and sexual spores exist for the spread of the fungus alone (e), which needs to associate with a photobiont. This photobiont may be obtained from different sources (f) such as another lichen, lichen propagules or free-living photobionts. In all these cases, the obtained photobiont may be compatible with the mycobiont (g) or not (h). If the photobiont is not compatible, the association will never proceed beyond the pre-thallus stage (i). However, this stage may provide a way of survival until a compatible photobiont is obtained (g). The fungal spore may also associate with a compatible photobiont from the beginning (j). After associating with a compatible photobiont, the development can proceed to a mature thallus (d). The type of signaling required to start the differentiation into a mature stratified thallus is yet not well established (Paulsrud, 2001; Joneson & Lutzoni, 2009; Joneson *et al.*, 2011).

In general, if the symbiotic-pairs are vertically transmitted, the association is maintained for many generations, but if they are horizontally transmitted the association is decoupled and must be restored after fungal reproduction. However, it is important to notice that even in the case of asexual reproduction, separation of phototrophic and fungal partners may occur, allowing co-dispersed phototrophic partners to be replaced by others available in the environment, or located in nearby lichen associations (Friedl,

1987). The frequency of such photobiont substitution or "switching" (Piercey-Normore, 2006; Piercey-Normore & De Priest, 2001) in nature is unknown, but this strategy may provide a certain level of flexibility in early stages of symbiosis and, importantly, a mechanism for optimizing symbiotic composition in a local environment (Yahr *et al.*, 2006).

1.3. Ecological model of lichenization

The ecological and genetic factors determining the development of a successful lichen symbiosis are still poorly understood. A standard conceptual model relating these factors is summarized in Figure 2.

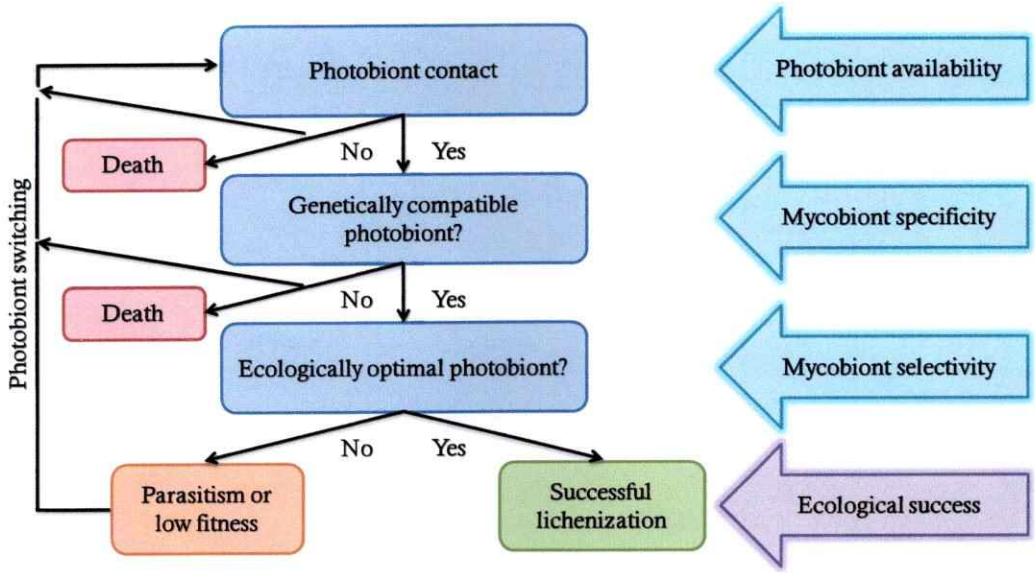


Figure 2. Ecological model of lichenization (see explanation in the text). The hypothetical model of mycobiont-photobiont association shows the interactions between partners and the mechanisms that determine them (modified from Yahr *et al.*, 2006).

After fungal spore germination, there must be a photobiont nearby with which to establish the symbiosis. The factor determining this event is known as *photobiont availability* and depends on geographical and ecological factors. If there is not a suitable photobiont available, most fungi will not be able to survive in the free living state.

Following the encounter between both partners, the successful establishment of the symbiosis requires a specific genetic compatibility between the mycobiont and the photobiont, factor known as *specificity*. Concerning this, lichen mycobionts have been normally considered as "specialists" because many have a high degree of specificity towards the photobionts they associate with; in contrast, lichen photobionts are less specific for their mycobionts, and therefore are normally referred to as "generalists" (Beck *et al.*, 2002; Yahr *et al.*, 2004; O'Brien, 2006; Otálora *et al.*, 2010). Even more, many different mycobiont species share the same photobiont, with only about 100 photobiont species reported to be associated with more than 13,500 lichen-forming fungal species (Lutzoni & Miadlikowska, 2009).

Another factor to consider in the establishment of the symbiosis is *selectivity*, which refers to a preferential association of the mycobionts with a subset of their compatible partners, i.e., if the mycobiont is presented with a choice of photobionts, it will preferentially associate with some over the others (Galun & Bubrick, 1984). Thus, the fungus is associated with only some of the available and compatible partners, forming with them stable associations more frequently than expected by chance (Ahmadjian, 1993; Honegger, 1993; Rambold *et al.*, 1998). It is important to mention that both specificity and selectivity refer to the symbiont association patterns, and are defined in this work from the mycobiont's perspective. This was done for simplicity since

mycobionts are thought to be more specific and dependent in their associations than photobionts, and does not reflect the amount of participation of either symbiont in selecting a partner.

Finally, the environmental fitness of the associations, or *ecological success*, is directly related to the abundance of the different symbiotic pairs, where the most abundant or "successful" pairs may reflect not only a high selectivity from the mycobiont towards those photobionts, but rather represent a combination of the above-mentioned factors.

It is not clear what drives selectivity, but there is a developing body of evidence suggesting that it depends on geographical and ecological factors (Wirtz *et al.*, 2003; Piercey-Normore, 2006; Yahr *et al.*, 2006, Škaloud & Peksa, 2010). Pinpointing the roles of the above-mentioned issues may help understand the ecological determinants of lichenization, evaluating the existence of an inter-relationship between the patterns of symbiosis and the geographic location of the species, their life histories, the environment in which they live, etc (Fedrowitz *et al.*, 2011).

1.4. Environmental influence on lichen associations

Patterns of symbiont diversity in lichens may be influenced by many interacting factors, including reproductive strategy, symbiont availability, the abiotic environment and the specific ecological requirements of each symbiont (Fedrowitz *et al.*, 2011). In fact, most of the current research focuses on the effect of the environment on the biodiversity and conservation of lichens, studying, for example, how changes in forest cover can have an effect in both, epiphytic and terricolous lichen species (Martínez *et*

al., 2011; Ramírez-Fernández *et al.*, 2013). There are several reports supporting the idea that forest degradation may influence the diversity of epiphytic lichen communities (i.e. those that grow on plant species) (e.g. Ellis *et al.*, 2007; Fritz *et al.*, 2008; Johansson 2008; Belinchón *et al.*, 2009; Aragón *et al.*, 2010; Otálora *et al.*, 2011; Király *et al.*, 2013), since alterations of forests lead to a reduction in the availability of their substrate, isolating the communities and changing microclimatic conditions (Kivistö & Kuusinen, 2000; Moen & Jonsson, 2003). Conversely, there is less information on the effects of forest degradation on terricolous lichens (i.e. soil-growing lichens), most of which address the effects of changes in land use due to agriculture and livestock (Scutari *et al.*, 2004; Motiejûnaitë & Faùtynowicz, 2005; St. Clair *et al.*, 2007; Rai *et al.*, 2012). In our previous work, Ramírez-Fernández *et al.* (2013), we showed that the diversity of *Peltigera* was higher in undisturbed environments like native forests of *Nothofagus* spp. than in degraded environments with no forest cover such as grasslands, indicating that decreasing forest quality also has a negative impact on the diversity of terricolous lichens. On the other hand, one of the most striking biogeographic patterns on the planet is the so called "latitudinal biodiversity gradient" (LBG), which suggests the existence of high species' numbers near the equator (at low latitudes) and low numbers of species at high latitudes. The gradient has been identified in almost all organisms that have been investigated, on land and in the sea (Dowle *et al.*, 2013); however, there are only few not very conclusive studies that address this issue in the case of symbiotic organisms such as lichens (e.g. Aragón *et al.*, 2012), despite the fact that they are considered to be almost ubiquitous in terrestrial ecosystems, and therefore constitute an ideal model system for investigating patterns of diversity such as the LBG.

Despite photobiont selection is a key factor in the development of lichens, there is still some controversy about it. Some authors (Rikkinen *et al.*, 2002; Dal Grande *et al.*, 2012; Dal Grande *et al.*, 2014) have pointed that the variation among lichen photobionts would be mainly the result of an evolutionary selection within and between ecological habitats through the existence of photobiont-mediated guilds, which consist on a common pool of photobionts that could potentially be forming a horizontally linked system. Nevertheless, Stenroos *et al.* (2006) and Myllys *et al.* (2007) concluded that mycobiont taxonomy was more important for the photobiont selection than habitat because mycobionts, at the species or genus level, could select restricted groups of photobionts across important habitat boundaries. However, several reports suggest that in temperate and tropical regions lichens have shown to be highly selective towards their photobionts (Helms *et al.*, 2001; Beck *et al.* 2002; Yahr *et al.*, 2004; Hauck *et al.*, 2007), while in more extreme habitats they seem to show lower selectivity (Romeike *et al.*, 2002; Wirtz *et al.*, 2003; Domaschke *et al.*, 2012; Pérez-Ortega *et al.*, 2012), suggesting that under these conditions there might be a mix between low availability of the photobionts and environmental limiting factors that would favor more versatile (less selective) mycobionts.

1.5. Study model

The biological model used in this study corresponds to lichens within the genus *Peltigera,* a cosmopolitan genus with an estimated number of 60–75 taxa (Goffinet & Hastings, 1995; Kirk *et al.*, 2001; Vitikainen, 2004). It has been mainly revised in the Northern Hemisphere (e.g. Goward *et al.*, 1995; Miadlikowska & Lutzoni, 2004), with

most species recorded from Europe and North America; and in some parts of the Southern Hemisphere like New Zealand (Galloway, 2000; Vitikainen, 2004; Galloway, 2007), Australia (Louwhoff, 2008) and, recently, Southern Chile (Martínez *et al.*, 2003, Quilhot *et al.*, 2012; Ramírez-Fernández *et al.*, 2013), a diverse region that remains poorly-studied in lichenological terms. However, most of these studies were regionally based and, therefore, knowledge of this genus is still geographically heterogeneous.

Peltigera lichens commonly occur in humid, mainly shaded habitats, on the forest floor, or along roadsides or other disturbed environments. They are predominantly terricolous or muscicolous (i.e., living on or in the soil or mosses, respectively), rarely saxicolous or corticolous (i.e., growing or living on rocks or tree barks, respectively). Galloway (2000, 2007) noted that species from this genus grow rapidly and often occur in disturbed habitats where they have a comparatively short life span.

Symbiotic entities within this genus are represented by two different types of associations: (1) bipartite symbioses involving a fungus and a cyanobacterium (*Nostoc*) and (2) tripartite symbioses involving a fungus, a green alga (*Coccomyxa*) as the main photobiont and a cyanobacterium (*Nostoc*) located in external cephalodia on the upper or lower surface of the thallus (Lutzoni & Miadlikowska, 2009). Although it is readily recognized in the field through the identification of some distinctive morphological characteristics (Figure 3), the genus is a taxonomically complex group, and many challenges remain at the species level (Goffinet & Hastings, 1995; Goward *et al.*, 1995; Miadlikowska & Lutzoni, 2000; Miadlikowska *et al.*, 2003). Among the most important of these characteristics are: "cephalodia" (internal or external structure of the thallus containing cyanobacteria in tripartite lichens), "apothecia" (sexual reproductive

structures of Ascomycota), "soredia" and "isidia" (asexual propagules), "rhizines" (root-like clusters of hyphae adapted for attaching a thallus to its substrate) and "veins" (strands of strengthening tissue on the lower surface of some lichens) (Goward *et al.*, 1994) (Figure 3).

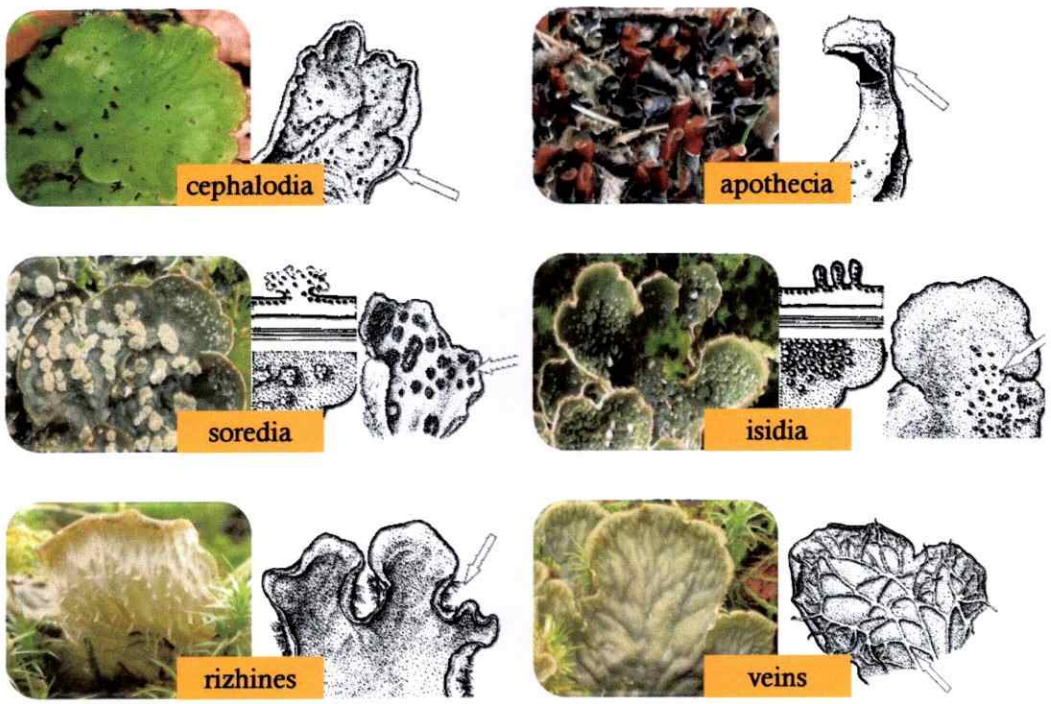


Figure 3. Main macroscopic structures considered in the recognition of *Peltigera* lichens (schematic representations modified from Goward *et al.*, 1994).

In any case, *Peltigera* is one of the most extensively studied lichen genera, and so is a valuable subject by which to explore the factors involved in the lichenization process and the distribution features of a widely distributed lichen genus (Martínez *et al.*, 2003).

Hypothesis

Given that (i) decreasing forest quality has a negative impact on lichen diversity, (ii) species diversity is lower at high latitudes than at low latitudes and (iii) the selection carried out by a mycobiont over the range of its specific available cyanobionts depends on the environmental context, being less selective in more adverse environments, it is proposed that: "Mycobionts within the genus *Peltigera* will be more selective for their specific cyanobionts in forested matrices than in un-forested ones and, in the case of the former, will be more selective at lower latitudes".

General Objective

Compare the selectivity of different *Peltigera* mycobionts in forested and un-forested matrices at different latitudes in Southern Chile and Antarctica considering the factors involved in the lichenization process.

Specific Objectives

1. Determine the *diversity* of the mycobionts and cyanobionts of *Peltigera* lichens per matrix type and sampling site.
2. Determine the *availability* of the potential cyanobionts per matrix type and sampling site.
3. Calculate the local *specificity* of the mycobionts and the *ecological success* of the holobionts per matrix type and sampling site.
4. Calculate the *selectivity* of the mycobionts for their available specific cyanobionts per matrix type and sampling site.

MATERIALS AND METHODS

2.1. Study Areas

The four sampling sites of this study were located in Southern Chile and Antarctica (Figure 4).

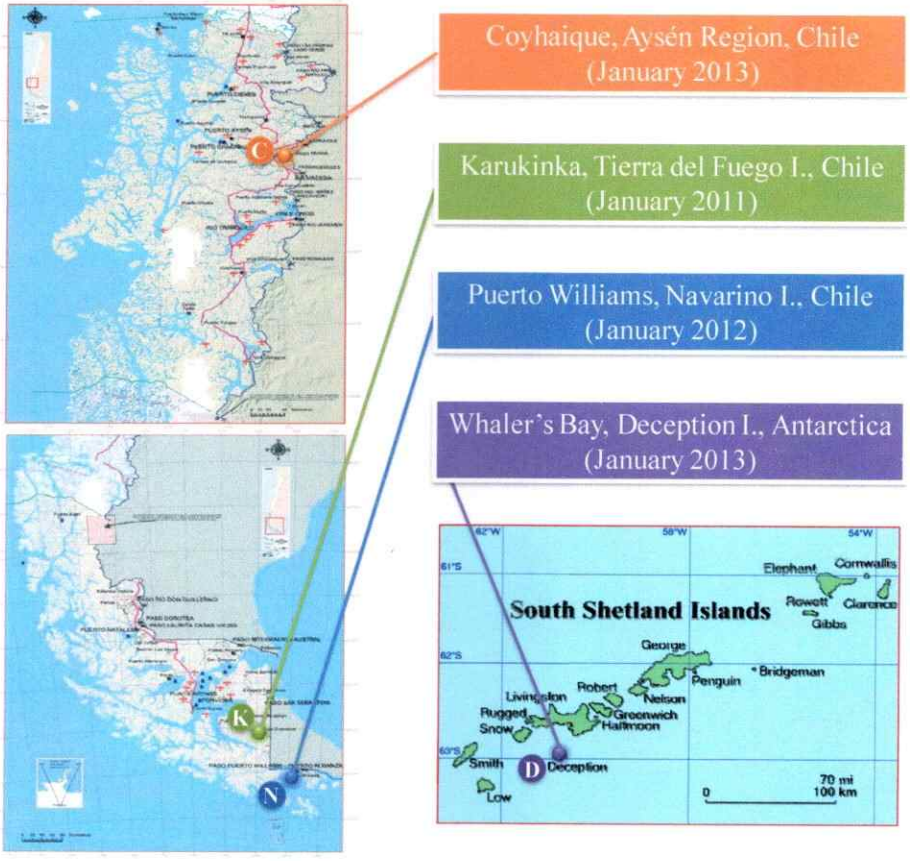


Figure 4. Sampling sites. The locations of the four sampling sites are shown as colored circles in the map. C: Coyhaique, K: Karukinka, N: Navarino, D: Deception. The date of each sampling is indicated in parenthesis.

In each of these sites, different sub-sites were established for performing the samplings, which were classified into forest-covered or uncovered matrices, according to the presence or absence of a forest cover, respectively (Table 1).

Table 1. Sampling sites and sub-sites.

Sampling sites	Sub-sites[a]	Matrix type	N° lichen samples	N° substrate samples
Coyhaique (C)	CF1	Forested	26	26
	CF2	Forested	25	25
Karukinka (K)	KY	Forested	20	20
	KM	Forested	20	20
	KG	Uncovered	20	20
Navarino (N)	NY	Forested	20	20
	NM	Forested	20	20
	NG	Uncovered	20	20
Deception (D)	DH	Uncovered	15	15

[a]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, DR: Deception Hillside.

2.1.1. Site 1: Coyhaique

This sampling was performed on January 2013 at the National Reserve of Coyhaique, Aysén Region, Chile (45° 31' 42.96" S, 72° 1' 51.95" W; orange circle, Figure 4); hereinafter referred to as "Coyhaique" or "C". This reserve is characterized by the presence of pure native forests of "lenga" (*Nothofagus pumilio* [Poepp. et Endl.] Krasser), and mixed native forests of "lenga-coigüe" (*N. pumilio* - *N. dombeyi* [Mirb.] Oerst.) or "lenga-ñirre" *(N. pumilio* - *N. antárctica* [G. Forst.] Oerst) (Till-Bottraud *et al.*, 2012). However, an important area of this reserve is also covered with plantations of exotic species of pines, such as *Pinus ponderosa* Douglas ex C. Lawson, *P. sylvestris* L.,

Larixdeciduas Mill. and *Pseudotsuga menziesii* (Mirb.) Franco (Quezada & Osorio, 2011).

In this location, two sub-sites were chosen for the collection of the samples, corresponding to young-forests of *N. pumilio*, which were named Coyhaique Forest 1 (CF1) and Coyhaique Forest 2(CF2). A total of 51 lichens and their associated substrates were collected from Coyhaique (Table 1).

2.1.2. Site 2: Karukinka

This sampling was performed on January 2011 in "Estancia Vicuña" within Karukinka Natural Park, Tierra del Fuego Island, Chile (54° 7' 51.67" S, 68° 42' 33.96" W; green circle, Figure 4); hereinafter referred to as "Karukinka" or "K"). The flora of this park is characterized by remnants of primary forests of "lenga" (*N. pumilio*), composite forests of "lenga-ñirre" (*N. pumilio-N. antarctica*) and a variety of other ecosystems (Arroyo *et al.*, 1996; Saavedra *et al.*, 2006).

Based on their forest cover, or the lack of it, three different sub-sites were chosen for collecting the samples: i) one young-forest of *N. pumilio* (KY), ii) one mature-forest of *N. pumilio* (KM) and iii) one grassland with no forest cover (KG).

From each of these sub-sites, a total of 20 lichen samples were taken along with their associated substrates, giving a final number of 60 lichen and 60 substrate samples for Karukinka (Table 1).

2.1.3. Site 3: Navarino

The sampling at this site was performed on January 2012, near to the locality of Puerto Williams, in Navarino Island, Cape Horn, Chile (54° 56' 33.71"S, 67° 37' 42.36"W; blue circle, Figure 4); hereinafter referred to as "Navarino" or "N". The flora of this area is characterized by the presence of both deciduous and ever-green forests where different species of *Nothofagus* are predominant, along with other ecosystems such as peatlands, wetlands, alpine zones and a variety of shrubby communities (Rozzi *et al.*, 2007). As in the case of Karukinka, three different sub-sites were chosen for collecting the samples, based on their forest cover or the lack of it: i) one young-forest of *N. pumilio* (NY), ii) one mature-forest of *N. pumilio* (NM) and iii) one grassland with no forest cover (NG).

From each sub-site a total of 20 lichen samples were taken along with their associated substrates, giving a final number of 60 lichen and 60 substrate samples for Navarino (Table 1).

2.1.4. Site 4: Deception

The sampling at this site was performed on January 2013 at Whaler's Bay, located in Deception Island, South Shetland Islands, Antarctica (62° 58' 22.22" S, 60° 34' 32.55" W; violet circle, Figure 4); hereinafter referred to as "Deception" or "D". This island contains a series of species classified as rare or extremely rare in the area, representing 25%, 17% and around 4% of the total mosses, agrimonies and lichens, respectively, known in Antarctica (ASPA 140, 2005).

A single sub-site was chosen for sampling, since it was the only place where *Peltigera* lichens were found in the island. It corresponded to a volcanic hillside and was named DH. The surfaces of this site are currently colonized by numerous species of mosses, agrimonies and lichens, many of which are extremely rare in Antarctica, including *Peltigera didactyla* (ASPA 140, 2005).

A total of 15 lichen samples and their associated substrates were collected, giving a final number of 15 lichen samples and 15 substrate samples for Deception (Table 1).

2.2. Sample collection

A photographic database of *Peltigera* lichens was constructed previous to the samplings, in order to recognize the lichen specimens *in situ* through the comparison and identification of the main macroscopic structures that characterize this lichen genus (See *Introduction*, Figure 3).

As aforementioned, a total of 186 lichens and their respective substrate samples were collected from the 4 sampling sites: 51 from Coyhaique, 60 from Karukinka, 60 from Navarino and 15 from Deception (Table 1). Samples consisted in a fragment of each lichen thallus, as well as a portion of the substrate adjacent to them. Both lichens and substrates were transported in paper bags to avoid their deterioration and reduce the moisture, and at low temperature inside cooler recipients to prevent microbial growth. Lichens were re-placed in clean paper bags at room temperature until use, while the soil samples were sieved and stored in plastic tubes at 4°C until use.

All lichens and sampling sites were photographed and geo-referenced (See Tables S1 and S2 on the *supplemental material*).

2.3. Pre-treatment of the samples and DNA extraction

2.3.1. Lichen samples

A thallus fragment from each sample was superficially cleaned with a sterile brush and spatula, thoroughly rinsed with sterile distilled water and air dried at room temperature. Eighty to 100 mg of each cleaned fragment were mechanically crushed with a mini-grinder, and DNA was extracted using the PowerSoil™ DNA Isolation kit (MoBio Laboratories Inc., CA, USA) according to the manufacturer's instructions.

2.3.2. Substrate samples

A total of 100 mg of the substrate samples from each sub-site were combined to generate composite samples, in order to decrease spatial heterogeneity. For example, 100 mg of each of the 20 samples from KY were combined into a single KY composite sample. The same procedure was done for the rest of the sub-sites, giving a final number of 9 composite samples (CF1, CF2, KY, KM, KG, NY, NM, NG and DH).

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 every extracted DNA, from lichens and substrates, 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 at -20°C until analysis.

2.4. PCR amplifications and sequencing

2.4.1. Mycobionts

From the isolated DNA of each lichen thallus, fungal LSU rDNA (large sub-unit of the ribosomal DNA) was amplified with specific primers LIC24R (Miadlikowska & Lutzoni, 2000) and LR7 (Vilgalys & Hester, 1990). In the case of one mycobiont (M3, see *Results* section 3.1.), the ITS (internal transcribed spacer) region (ITS1-5.8S-ITS2) was also amplified using fungal primers ITS1 and ITS4 (White *et al.*, 1990) (Table 2).

2.4.2. Cyanobionts

From the isolated DNA of each lichen thallus, the cyanobacterial SSU rDNA (small sub-unit of the ribosomal DNA) was amplified with primers PCR1 and PCR18 (Wilmotte *et al.*, 1993) (Table 2).

On the other hand, from the isolated DNA of each composite substrate sample, cyanobacterial SSU rDNA was amplified using the primers *Cya*106F and *Cya*781R (Nübel *et al.*, 1997) (Table 2).

Table 2. Primer pairs used to amplify ribosomal genes (cites in the text).

Primers	Nucleotide sequence (5'-3')	Expected size (bp)
LIC24R	GAAACCAACAGGGATTG	1200
LR7	TACTAC CACCAAGATCT	
ITS1	TCCGTAGGTGAACCTGCGG	700
ITS4	TCCTCCGCTTATTGATATGC	
PCR1	AGAGTTTGATCCTGGCTCAG	1500
PCR18	TTTGCGGCCGCTCTGTGTGCCTAGGTATCC	
*Cya*106F	CGGACGGGTGAGTAACGCGTGA	600
*Cya*781R	GACTACTGGGGTATCTAATCCCATT	

PCR conditions for each primer set are specified in Table 3. All reactions were prepared in a 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 run in a Maxygene thermocycler (Axygen, CA, USA).

Table 3. PCR conditions for each primer set.

N° cycles→	1 cycle		30 cycles						1 cycle	
Primer pairs	In. Denat.		Denat.		Anneal.		Extens.		Fin. Extens.	
LIC24R-LR7	94°C	1:00	94°C	0:30	52°C	0:30	72°C	1:30	72°C	4:00
ITS1-ITS4	94°C	3:00	94°C	1:00	60°C	1:00	72°C	1:00	72°C	7:00
PCR1-PCR18	94°C	1:00	94°C	1:00	55°C	1:00	72°C	4:00	72°C	7:00
*Cya*106F-*Cya*781R	94°C	5:00	94°C	1:00	60°C	1:00	72°C	1:00	72°C	10:00

In. Denat: initial denaturation temperature and time, Denat.: denaturation temperature and time, Anneal.: annealing temperature and time, Extens.: extension temperature and time, Fin. Extens.: final extension temperature and time.

Concentration and quality of the amplicons were determined electrophoretically as described above, except that 1.2 % (w/v) agarose gels were used.

Amplicons obtained from the lichen samples were sequenced in one direction (Macrogen Inc., Seoul, South Korea) using the forward primers in a Genetic Analyzer 3730XL (Applied Biosystems, CA, USA). All sequences obtained from the lichen samples were deposited in the GenBank database under accession numbers KF718515 to KF718640 and KC514744 to KC514803 (fungal LSU rDNA); KF718389 to KF718514 and KC514624 to KC514683 (cyanobacterial SSU rDNA) (see Table S1 in the Supporting Information for additional details).

For each of the 9 composite substrate samples described in section 2.3.2., the cyanobacterial SSU rDNA amplicons obtained with primers *Cya*106F-*Cya*781R were cloned and sequenced by the Library Construction and Sequencing Service provided by Macrogen (Macrogen Inc., Seoul, South Korea), generating a total of 9 clone libraries with 96 sequenced clones each.

2.5. Sequence Analyses

DNA sequences were visually checked and manually edited on Mega 5.2 software (Tamura *et al.*, 2011) and aligned with the Muscle alignment tool (Edgar, 2004) provided in the same software. The presence of ambiguously aligned nucleotides was checked on the web server Guidance (Penn *et al.*, 2010) and removed prior to the following analyses.

Edited sequence fragments from both mycobionts and cyanobionts were subjected to blast-n queries (Altschul *et al.*, 1990) for an initial verification of their identities by comparison to the non-redundant nucleotide database at GenBank (NCBI).

2.6. Phylogenetic Analyses

2.6.1. Mycobionts

The mycobiont operational taxonomic units (OTUs) were defined as those groups of sequences that were 100% identical (nucleotide identity), that is, no different sequences were included in the same OTU.

The phylogenetic analyses were performed on a fungal LSU rDNA sequence set consisting of one representative of each of the different mycobiont OTUs (*i.e.*, no repeated sequences were included) plus a selection of 67 *Peltigera* accessions selected from some previous works on this genus (Miadlikowska & Lutzoni, 2000; Miadlikowska *et al.* 2003; Goffinet *et al.*, 2003) and downloaded from GenBank. The accessions included one representative of the sequenced species reported in the above-mentioned works (not including the Hydrotheriae clade; Miadlikowska *et al.* 2014) and, if the same species was reported in more than a single geographic site, representatives of each site where included in the selection.

Maximum likelihood (ML) analyses were performed on the T-REX web server (Boc *et al.*, 2012) under the PhyML algorithm. The best nucleotide substitution model was determined with the help of jModelTest 2.1.1. (Posada, 2008) under the corrected Akaike Information Criteria (AICc), which suggested the TIM2+I+G as the best fitting model of evolution for the ML analyses. Because this model cannot be implemented in PhyML, the data set was analyzed using the GTR+I+G model of evolution, since it was the closest model available in PhyML.

Bayesian Inference (BI) was carried out based on the same model of evolution selected and using the Metropolis-coupled Bayesian Markov chain Monte Carlo

algorithm $(MC)^3$ implemented in the software MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Four independent runs of 10 million generations each were made, sampling the chains every 1000 generations. The first 2500 samples were discarded as burn-in, and convergence was assessed by examining all parameters using Tracer v. 1.5 (Rambaut *et al.*, 2014, available from http://beast.bio.ed.ac.uk/Tracer).

In the fungal analyses *Solorina saccata* isolate AFTOL-ID 127 was used as outgroup (accession number DQ973044). OTUs were named based on statistically supported nodes (Bootstrap >75% and PP >0.95), recovered in the phylogenetic analysis.

2.6.2. Cyanobionts

In the case of the cyanobionts, OTUs were defined under the same criteria used for the mycobionts, that is, each OTU was comprised of identical sequences. ML and BI phylogenetic reconstructions were performed on a cyanobacterial SSU rDNA sequence set which consisted of one representative of each cyanobacterial OTU plus 49 *Nostoc* SSU rDNA sequences downloaded from GenBank for comparison. These sequences included a selection of the *Nostoc* sequences reported by O'Brien *et al.* (2005), in addition to close matches to our cyanobionts according to the blast-n results, if they were not already included in the selection from O'Brien *et al.* (2005). Both phylogenetic analyses were performed as described in the case of the mycobionts, except that the TPM2uf+I+G was selected as the best nucleotide substitution model. This model cannot be implemented in PhyML, so the data set was analyzed using the GTR+I+G model of evolution as it was the most similar model available in PhyML.

In the cyanobacterial analyses *Fischerella muscicola* strain PCC7414 (accession number AF132788) was set as outgroup.

All phylogenetic trees were drawn on the program TreeGraph 2.0.54-364 beta (Stöver& Müller, 2010). In addition, in order to graph the relationship between the cyanobiont OTUs with which each mycobiont associates, the Median Joining algorithm in the Network v4.6 program (Bandelt *et al.*, 1999) was used.

2.7. Indices Calculation

2.7.1. Diversity Indices

The diversity of the mycobionts and the lichenized cyanobionts in each sub-site was calculated with the Weighted Shannon's Diversity Index (WSD) (Casquilho *et al.*, 1997) as in [1], with p_i the frequency of each symbiont and α_i their relatedness according to [2], defined in this study as the ratio of the average p-distance between the analyzed symbionts and the maximum distance achievable under the genus level, i.e., 0.05 (Nemergut *et al.*, 2011).

$$WSD = -\sum \alpha_i p_i log p_i \quad [1]$$

$$\alpha_i = \frac{d_i}{d_{max}} \quad [2]$$

This maximum distance was chosen because the lichens from the present study (*Peltigera* spp.) always associate with the cyanobacterial genus *Nostoc* and, therefore, the maximum distance that can exist between them is always under the genus level.

The potential available cyanobionts (*(a)Cs*) included the abundance of the non-lichenized cyanobionts in the substrates of each sub-site (*(s)Cs*) plus the lichenized cyanobionts of each sub-site (*(l)Cs*) according to [3]. Finally, the diversity of the potential available cyanobionts was determined with the *WSD*, as described in [1] for the mycobionts and lichenized cyanobionts.

$$(a)Cs = (s)Cs + (l)Cs \qquad [3]$$

2.7.2. Specificity Indices

The specificity of each mycobiont was defined under the concept of *Local Phylogenetic Specificity* (Poulin *et al.*, 2011), which considers the number of discrete cyanobiont OTUs they were associated with, weighted by their relatedness. The formula designed and utilized for calculating this factor is described in *Results*, section 3.5.

2.7.3. Selectivity Indices

Selectivity (Se) was theoretically defined as the preferential association of the mycobionts with some of their specific cyanobionts and, therefore, it did not only consider the number of different cyanobionts and their relatedness, but also the frequency of the different symbiotic pairs formed by each mycobiont and the availability of the potential specific cyanobionts. It was calculated for each mycobiont using a modified version, proposed in this study, of Vanderploeg & Scavia's elegibility index (Vanderploeg & Scavia, 1979), as is described in *Results*, section 3.7.

2.8. Statistical Tests

Bootstrap tests of 10,000 re-sampling randomizations were performed in the computer software R-studio version 0.98.501 (available at http://www.rstudio.org) for determining the confidence intervals in all the comparisons of diversity, ecological success and selectivity per matrix type and per sampling site. Overlapping intervals were considered not statistically significant. Finally, a G-test was performed in the computer software R-studio version 0.98.501 for comparing the observed versus the expected abundances of the symbiotic pairs and determining if the symbiotic associations were specific.

RESULTS

3.1. Operational taxonomic units (OTUs) definition

3.1.1. Mycobionts

One hundred and eighty-six fungal LSU rDNA sequences were successfully amplified from the DNA extracted directly from the lichen samples. A blast-n analysis on these sequences confirmed that they all belonged to the genus *Peltigera*. Among the 186 amplicons, 8 different OTUs were established and named from M1 to M8. The morphological characteristics analyzed confirmed these groupings since morphology differed among them, but was consistent within each group.

In order to determine the relation of these specimens with other *Peltigera* lichens from the rest of the world, a sequence set was created consisting of one representative of each group plus the 67 LSU rDNA *Peltigera* sequences downloaded from GenBank, giving a final set of 76 sequences with 728 nucleotide positions each. This sequence set was subjected to ML and BI analyses and, given that both yielded similar tree topologies, only the best tree obtained from the ML is shown (Figure 5).

Results

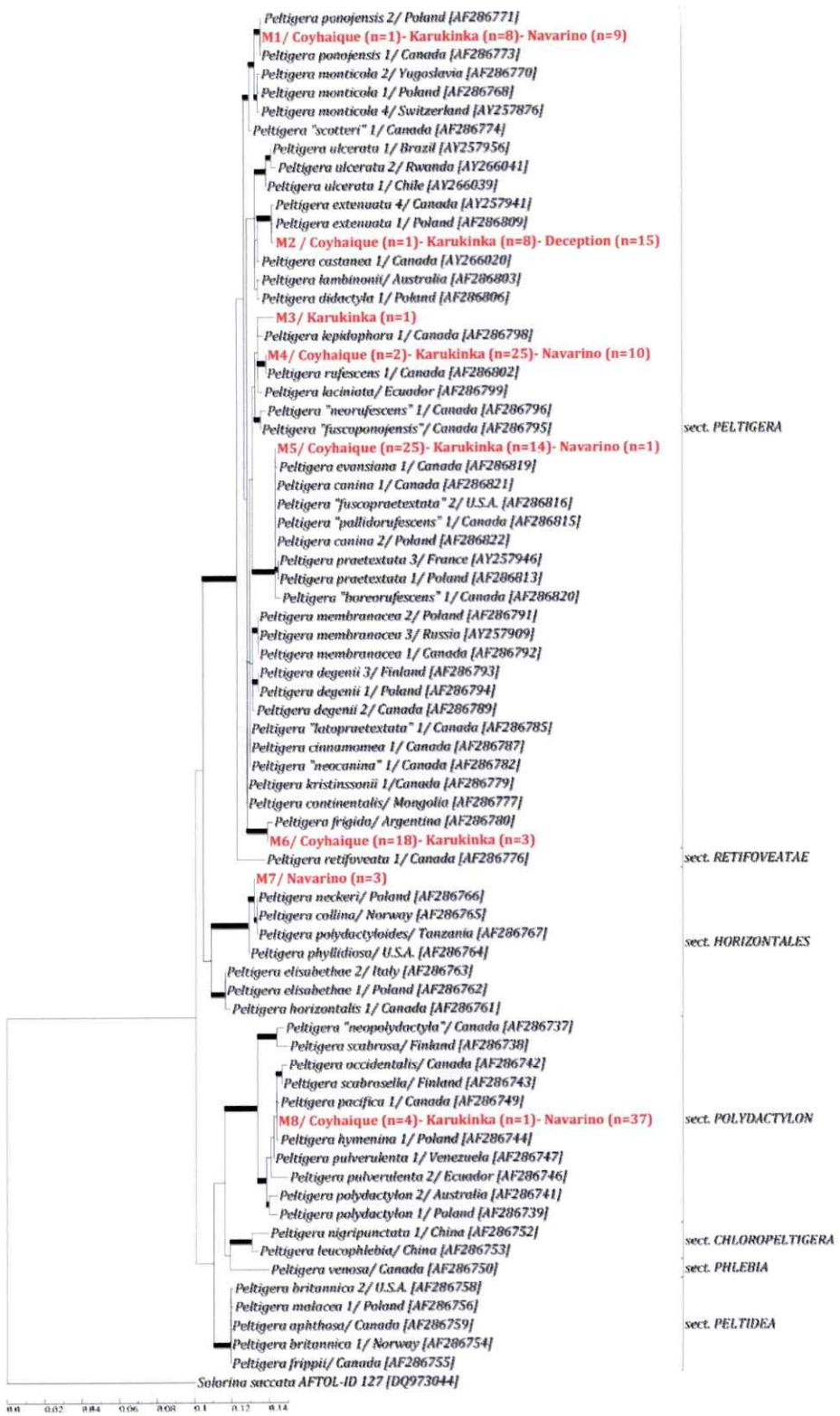

Peltigera ponojensis 2/ Poland [AF286771]
M1/ Coyhaique (n=1)- Karukinka (n=8)- Navarino (n=9)
Peltigera ponojensis 1/ Canada [AF286773]
Peltigera monticola 2/ Yugoslavia [AF286770]
Peltigera monticola 1/ Poland [AF286768]
Peltigera monticola 4/ Switzerland [AY257876]
Peltigera "scotteri" 1/ Canada [AF286774]
Peltigera ulcerata 1/ Brazil [AY257956]
Peltigera ulcerata 2/ Rwanda [AY266041]
Peltigera ulcerata 1/ Chile [AY266039]
Peltigera extenuata 4/ Canada [AY257941]
Peltigera extenuata 1/ Poland [AF286809]
M2 / Coyhaique (n=1)- Karukinka (n=8)- Deception (n=15)
Peltigera castanea 1/ Canada [AY266020]
Peltigera lambinonii/ Australia [AF286803]
Peltigera didactyla 1/ Poland [AF286806]
M3/ Karukinka (n=1)
Peltigera lepidophora 1/ Canada [AF286798]
M4/ Coyhaique (n=2)- Karukinka (n=25)- Navarino (n=10)
Peltigera rufescens 1/ Canada [AF286802]
Peltigera laciniata/ Ecuador [AF286799]
Peltigera "neorufescens" 1/ Canada [AF286796]
Peltigera "fuscoponojensis"/ Canada [AF286795]
sect. PELTIGERA
M5/ Coyhaique (n=25)- Karukinka (n=14)- Navarino (n=1)
Peltigera evansiana 1/ Canada [AF286819]
Peltigera canina 1/ Canada [AF286821]
Peltigera "fuscopraetextata" 2/ U.S.A. [AF286816]
Peltigera "pallidorufescens" 1/ Canada [AF286815]
Peltigera canina 2/ Poland [AF286822]
Peltigera praetextata 3/ France [AY257946]
Peltigera praetextata 1/ Poland [AF286813]
Peltigera "boreorufescens" 1/ Canada [AF286820]
Peltigera membranacea 2/ Poland [AF286791]
Peltigera membranacea 3/ Russia [AY257909]
Peltigera membranacea 1/ Canada [AF286792]
Peltigera degenii 3/ Finland [AF286793]
Peltigera degenii 1/ Poland [AF286794]
Peltigera degenii 2/ Canada [AF286789]
Peltigera "latopraetextata" 1/ Canada [AF286785]
Peltigera cinnamomea 1/ Canada [AF286787]
Peltigera "neocanina" 1/ Canada [AF286782]
Peltigera kristinssonii 1/Canada [AF286779]
Peltigera continentalis/ Mongolia [AF286777]
Peltigera frigida/ Argentina [AF286780]
M6/ Coyhaique (n=18)- Karukinka (n=3)
Peltigera retifoveata 1/ Canada [AF286776]
sect. RETIFOVEATAE
M7/ Navarino (n=3)
Peltigera neckeri/ Poland [AF286766]
Peltigera collina/ Norway [AF286765]
Peltigera polydactyloides/ Tanzania [AF286767]
sect. HORIZONTALES
Peltigera phyllidiosa/ U.S.A. [AF286764]
Peltigera elisabethae 2/ Italy [AF286763]
Peltigera elisabethae 1/ Poland [AF286762]
Peltigera horizontalis 1/ Canada [AF286761]
Peltigera "neopolydactyla"/ Canada [AF286737]
Peltigera scabrosa/ Finland [AF286738]
Peltigera occidentalis/ Canada [AF286742]
Peltigera scabrosella/ Finland [AF286743]
Peltigera pacifica 1/ Canada [AF286749]
M8/ Coyhaique (n=4)- Karukinka (n=1)- Navarino (n=37)
sect. POLYDACTYLON
Peltigera hymenina 1/ Poland [AF286744]
Peltigera pulverulenta 1/ Venezuela [AF286747]
Peltigera pulverulenta 2/ Ecuador [AF286746]
Peltigera polydactylon 2/ Australia [AF286741]
Peltigera polydactylon 1/ Poland [AF286739]
Peltigera nigripunctata 1/ China [AF286752]
sect. CHLOROPELTIGERA
Peltigera leucophlebia/ China [AF286753]
Peltigera venosa/ Canada [AF286750]
sect. PHLEBIA
Peltigera britannica 2/ U.S.A. [AF286758]
Peltigera malacea 1/ Poland [AF286756]
Peltigera aphthosa/ Canada [AF286759]
sect. PELTIDEA
Peltigera britannica 1/ Norway [AF286754]
Peltigera frippii/ Canada [AF286755]
Solorina saccata AFTOL-ID 127 [DQ973044]
0.0
0.02
0.04
0.06
0.08
0.1
0.12
0.14

Figure 5. (previous page) Phylogenetic relationships among *Peltigera* mycobionts. Phylogeny was based on a maximum likelihood (ML) analysis of 72 LSU rDNA sequences which included 728 nucleotide positions. Support values are indicated as wide branches for nodes that received significant support (maximum-likelihood bootstrap values ≥75% or Bayesian Inference pp values ≥0.95). Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. The mycobionts from this study, named from M1 to M8, are shown in red. The geographical origin is also indicated for each mycobiont and the number of specimens per sampling site is shown between parentheses. The division of *Peltigera* species into sections (sect.) corresponds to the classification proposed by Miadlikowska & Lutzoni (2000). Taxa in quotation marks correspond to those that have not yet been formally published. Numbers in square brackets next to the sequences downloaded from the database correspond to their accession numbers. The geographical origin of these specimens is also shown next to their names, separated by a slash.

The analyses placed mycobionts M1 to M8 in different positions along the *Peltigera* phylogenetic tree (Figure 5, red names). According to the classification of this genus into "sections", proposed by Miadlikowska & Lutzoni (2000), samples from this study belonged to sections *Peltigera* (M1 to M6), *Horizontales* (M7) and *Polydactylon* (M8).

Within these sections, most of the mycobionts from this study formed defined and well-supported monophyletic groups with some of the other *Peltigera* species: M1 was closely related to *P. ponojensis*, M2 to *P. extenuata*, M4 to *P. rufescens* and M6 to *P. frigida*. The rest of the mycobionts were closely-related to lineages formed by more than one species: M5 to *P. evansiana* / *P. canina* / *P. "fuscopraetextata"* / *P. "pallidorufescens"* / *P. praetextata* / *P. "boreorufescens"* (hereinafter *P. canina* lineage), M7 to *P. neckeri* / *P. collina* / *P. polydactyloides* (hereinafter *P. neckeri* lineage), and M8 to *P. polydactylon* / *P. occidentalis* / *P. scabrosella* / *P. pacifica* / *P. hymenina* / *P. pulverulenta* (hereinafter *P. hymenina* lineage). M3 was the only mycobiont which was not closely related to any of the sequences downloaded from the database and, therefore, it was not classified beneath the section level. For that reason,

the ITS region was used as a second molecular marker. Despite several attempts to obtain high quality ITS sequences, this was only possible for a 200 nucleotide fragment of this region. A blast-n analysis showed, with a 98% nucleotide identity, that this specimen could be related to *P.* "*granulosa*" or *P.* "*papuana*", both described by Sérusiaux *et al.* (2009) as new species from New Guinea. Moreover, the ITS-HR (ITS Hyper-variable Region) (Miadlikowska *et al.*, 2003) was similar to the one proposed for the new putative group *P. "papuanorum"*, which includes both species and is briefly described by Lutzoni *et al.* (unpublished data [available at: http://www.peltigera.lutzonilab.net]). However, M3's ITS-HR was interrupted by an insert of 86 nucleotides, suggesting it might belong to a new, still undescribed, species.

Regarding the mycobiont distribution among the different sites, most (M1, M2, M4, M5 and M8) were present in up to 3 of the 4 sampling sites but none was found in all of them (Figure 5). The most abundant OTUs were M8 and M5, present in 42 and 40 specimens, respectively, from Coyhaique, Karukinka and Navarino. Other mycobionts also found in these 3 sites were M4 (n= 37) and M1 (n= 18). The only mycobiont present in the specimens from Deception was M2 (n= 24), which also had representatives in Coyhaique and Karukinka. M6 was present in Coyhaique (n= 18) and Karukinka (n= 3) and, finally, mycobionts M3 (n= 1) and M7 (n= 3) were found exclusively in a single geographic location, Karukinka and Navarino, respectively.

3.1.2. Cyanobionts

All 186 cyanobacterial SSU rDNA sequences were successfully amplified from the lichen-thallus samples. Every sequencing reaction produced clean reads with no

evidence of secondary peaks, suggesting that only a single photobiont genotype dominated in each lichen thallus. A blast-n analysis of these sequences confirmed that they all belonged to the genus *Nostoc*. Among the 186 amplicons, 15 different OTUs were established and named from C1 to C15.

In order to determine how these cyanobionts were related to other *Nostoc* strains from different parts of the world, a sequence set was created consisting of one representative of each of the 15 cyanobiont OTUs along with the 49 *Nostoc* SSU rDNA sequences downloaded from GenBank, giving a final set of 65 sequences with 690 nucleotide positions each. Phylogenetic analyses of ML and BI were performed on this sequence set and, since both yielded similar tree topologies, only the best tree obtained from ML is shown (Figure 6).

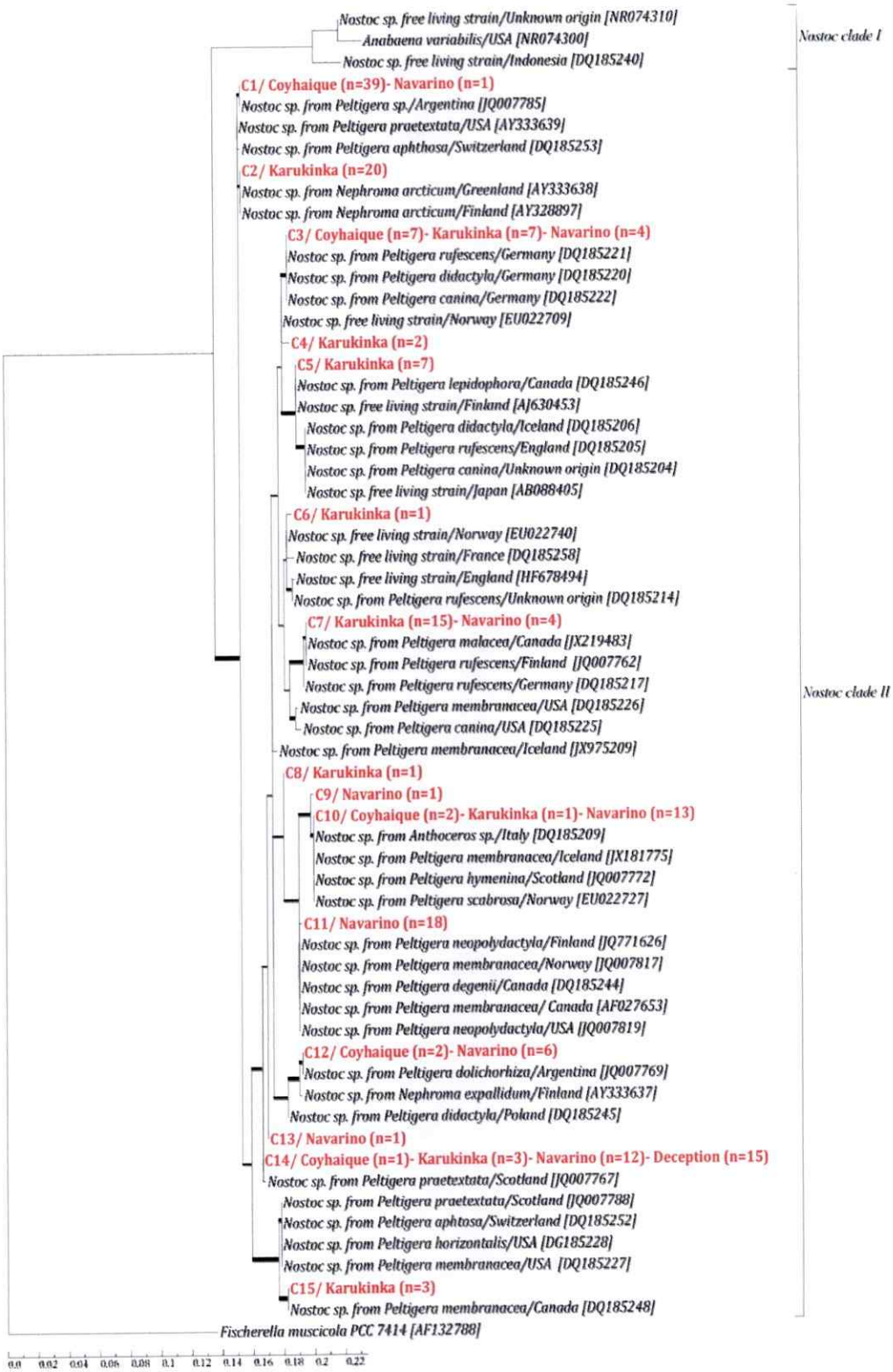

Nostoc sp. free living strain/Unknown origin [NR074310]
Anabaena variabilis/USA [NR074300]
Nostoc sp. free living strain/Indonesia [DQ185240]
Nostoc clade I
C1/ Coyhaique (n=39)- Navarino (n=1)
Nostoc sp. from Peltigera sp./Argentina [JQ007785]
Nostoc sp. from Peltigera praetextata/USA [AY333639]
Nostoc sp. from Peltigera aphthosa/Switzerland [DQ185253]
C2/ Karukinka (n=20)
Nostoc sp. from Nephroma arcticum/Greenland [AY333638]
Nostoc sp. from Nephroma arcticum/Finland [AY328897]
C3/ Coyhaique (n=7)- Karukinka (n=7)- Navarino (n=4)
Nostoc sp. from Peltigera rufescens/Germany [DQ185221]
Nostoc sp. from Peltigera didactyla/Germany [DQ185220]
Nostoc sp. from Peltigera canina/Germany [DQ185222]
Nostoc sp. free living strain/Norway [EU022709]
C4/ Karukinka (n=2)
C5/ Karukinka (n=7)
Nostoc sp. from Peltigera lepidophora/Canada [DQ185246]
Nostoc sp. free living strain/Finland [AJ630453]
Nostoc sp. from Peltigera didactyla/Iceland [DQ185206]
Nostoc sp. from Peltigera rufescens/England [DQ185205]
Nostoc sp. from Peltigera canina/Unknown origin [DQ185204]
Nostoc sp. free living strain/Japan [AB088405]
C6/ Karukinka (n=1)
Nostoc sp. free living strain/Norway [EU022740]
Nostoc sp. free living strain/France [DQ185258]
Nostoc sp. free living strain/England [HF678494]
Nostoc sp. from Peltigera rufescens/Unknown origin [DQ185214]
C7/ Karukinka (n=15)- Navarino (n=4)
Nostoc sp. from Peltigera malacea/Canada [JX219483]
Nostoc sp. from Peltigera rufescens/Finland [JQ007762]
Nostoc sp. from Peltigera rufescens/Germany [DQ185217]
Nostoc sp. from Peltigera membranacea/USA [DQ185226]
Nostoc sp. from Peltigera canina/USA [DQ185225]
Nostoc sp. from Peltigera membranacea/Iceland [JX975209]
C8/ Karukinka (n=1)
C9/ Navarino (n=1)
C10/ Coyhaique (n=2)- Karukinka (n=1)- Navarino (n=13)
Nostoc sp. from Anthoceros sp./Italy [DQ185209]
Nostoc sp. from Peltigera membranacea/Iceland [JX181775]
Nostoc sp. from Peltigera hymenina/Scotland [JQ007772]
Nostoc sp. from Peltigera scabrosa/Norway [EU022727]
C11/ Navarino (n=18)
Nostoc sp. from Peltigera neopolydactyla/Finland [JQ771626]
Nostoc sp. from Peltigera membranacea/Norway [JQ007817]
Nostoc sp. from Peltigera degenii/Canada [DQ185244]
Nostoc sp. from Peltigera membranacea/ Canada [AF027653]
Nostoc sp. from Peltigera neopolydactyla/USA [JQ007819]
C12/ Coyhaique (n=2)- Navarino (n=6)
Nostoc sp. from Peltigera dolichorhiza/Argentina [JQ007769]
Nostoc sp. from Nephroma expallidum/Finland [AY333637]
Nostoc sp. from Peltigera didactyla/Poland [DQ185245]
C13/ Navarino (n=1)
C14/ Coyhaique (n=1)- Karukinka (n=3)- Navarino (n=12)- Deception (n=15)
Nostoc sp. from Peltigera praetextata/Scotland [JQ007767]
Nostoc sp. from Peltigera praetextata/Scotland [JQ007788]
Nostoc sp. from Peltigera aphtosa/Switzerland [DQ185252]
Nostoc sp. from Peltigera horizontalis/USA [DG185228]
Nostoc sp. from Peltigera membranacea/USA [DQ185227]
C15/ Karukinka (n=3)
Nostoc sp. from Peltigera membranacea/Canada [DQ185248]
Nostoc clade II
Fischerella muscicola PCC 7414 [AF132788]
0.0 0.02 0.04 0.06 0.08 0.1 0.12 0.14 0.16 0.18 0.2 0.22

Figure 6. (previous page) Phylogenetic relationships among *Nostoc* cyanobionts. Phylogeny was based on maximum likelihood (ML) analyses of 64 SSU rDNA sequences which included 690 nucleotide positions. Support values are indicated for nodes that received significant support (maximum-likelihood bootstrap values ≥75% or Bayesian Inference pp values ≥0.95). Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. The cyanobionts from this study, named from C1 to C15, are shown in red. The geographical origin is also indicated for each cyanobiont and the number of specimens per sampling site is shown between parentheses. The division of the species into *Nostoc* clades I and II corresponds to the classification proposed by O'Brien *et al.* (2005). Numbers in square brackets next to the sequences downloaded from the database correspond to their accession numbers. The geographical origin of these specimens is also shown next to their names, separated by a slash.

The analyses showed that all the cyanobionts from this work belong to *Nostoc* II clade proposed by O'Brien *et al.* (2005), and *Peltigera* guild proposed by Rikkinen *et al.* (2002) (data not shown).

All the *Nostoc* sequences reported in this study were related (>97.5% nucleotide identity) to cyanobionts previously found in other lichens such as *Peltigera* spp. and *Nephroma* spp. from North America and Europe, except C6, which was more related to European free-living *Nostoc* strains, and C1 and C12, more closely-related to cyanobionts reported from South American *Peltigera* specimens.

The most abundant cyanobiont OTU was C1, with 40 samples corresponding to specimens from Coyhaique and Navarino. The second largest OTU was C14 present in 31 samples, which was also the only cyanobiont found in the 4 sampling sites. C3 (n= 18) and C10 (n= 16) were found in Coyhaique, Karukinka and Navarino specimens; and C12 (n= 8) and C7 (n= 19) had representatives from Coyhaique-Navarino and Karukinka-Navarino, respectively. The remaining OTUs consisted of sequences from a single sampling site; 6 types were exclusively represented by sequences from Karukinka

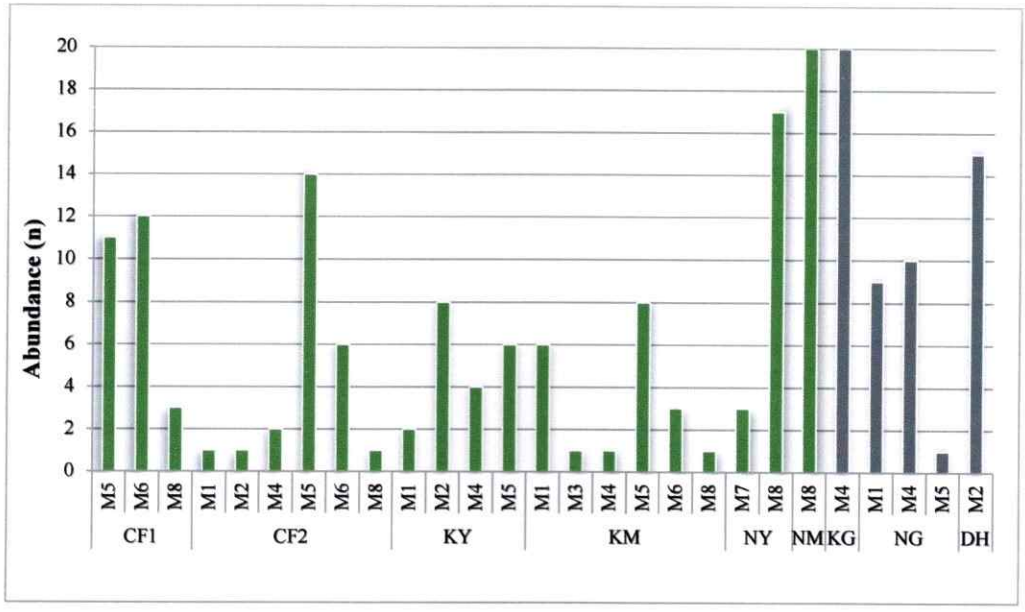
(C2, n= 20; C4, n= 2; C5, n= 7; C6, n= 1; C8, n= 1; C15, n= 3) and 3 by sequences exclusively from Navarino (C9, n= 1; C11, n= 18; C13, n= 1).

3.2. Symbiont diversity

There were 9 different sub-sites within the 4 sampling sites, 6 forested and 3 un-forested, and the abundance of each symbiont type in these sub-sites is shown in Figure 7. According to the Weighted Shannon Diversity index (WSD), which considers the number of OTUs present in a determined site, their relative abundance and the genetic relation between them, the diversity of both symbiotic components was calculated in each of these 9 sub-sites, and were then ranked according to their diversity (WSDR), as shown in Table 4.

It can be observed that the diversity of the mycobionts was, in general, higher than that of the lichenized cyanobionts. The mycobionts were more diverse in KM followed by CF1 and CF2, all forested environments, while in the case of the cyanobionts the most diverse sub-sites were KM and NG, one forested and one un-forested matrix, respectively. In both cases, the least diverse sub-sites were DH, KG and NM.

A.



B.

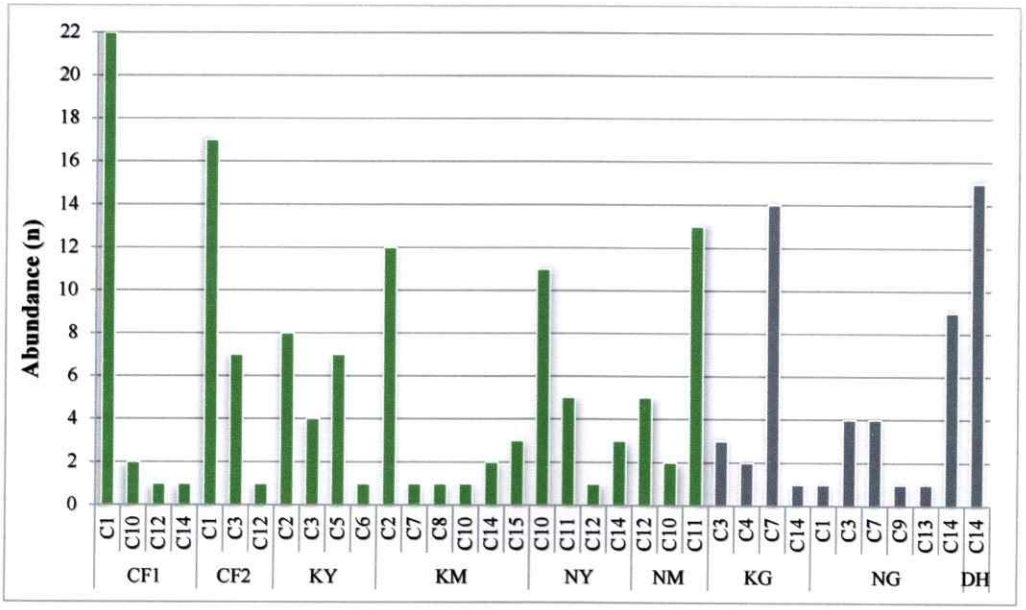


Figure 7. Symbiont abundance in the different sub-sites. A. Mycobionts (M1-M8). B. Cyanobionts (C1-C14). Forested matrices (green columns): Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices (gray columns): Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).

Table 4. Symbiont diversity per sub-site.

		Ms[b] Diversity		*(l)Cs*[c] Diversity	
	Sub-sites[a]	**WSD**[d]	**WSDR**[e]	**WSD**	**WSDR**
Forested	CF1	0.780	II	0.190	VI
	CF2	0.691	III	0.243	V
	KY	0.461	IV	0.299	III
	KM	0.821	I	0.432	I
	NY	0.430	V	0.289	IV
	NM	0.000	VII	0.157	VII
Un-forested	KG	0.000	VII	0.133	VIII
	NG	0.329	VI	0.330	II
	DH	0.000	VII	0.000	IX

[a]Forested matrices: Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices: Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).
[b]*Ms*: mycobionts.
[c]*(l)Cs*: lichenized cyanobionts.
[d]WSD: Weighted Shannon Diversity.
[e]WSDR: Weighted Shannon Diversity Rank. I: most diverse; IX: least diverse.

In order to determine the effect of the matrix type on the diversity of the symbionts, their average diversity was calculated grouping the different sub-sites according to their forest cover (Figure 8). As can be observed, the mycobionts were more diverse in the forested matrices than in the un-forested ones (Figure 8A), while the cyanobionts presented similar diversity values in both matrix types (Figure 8B).

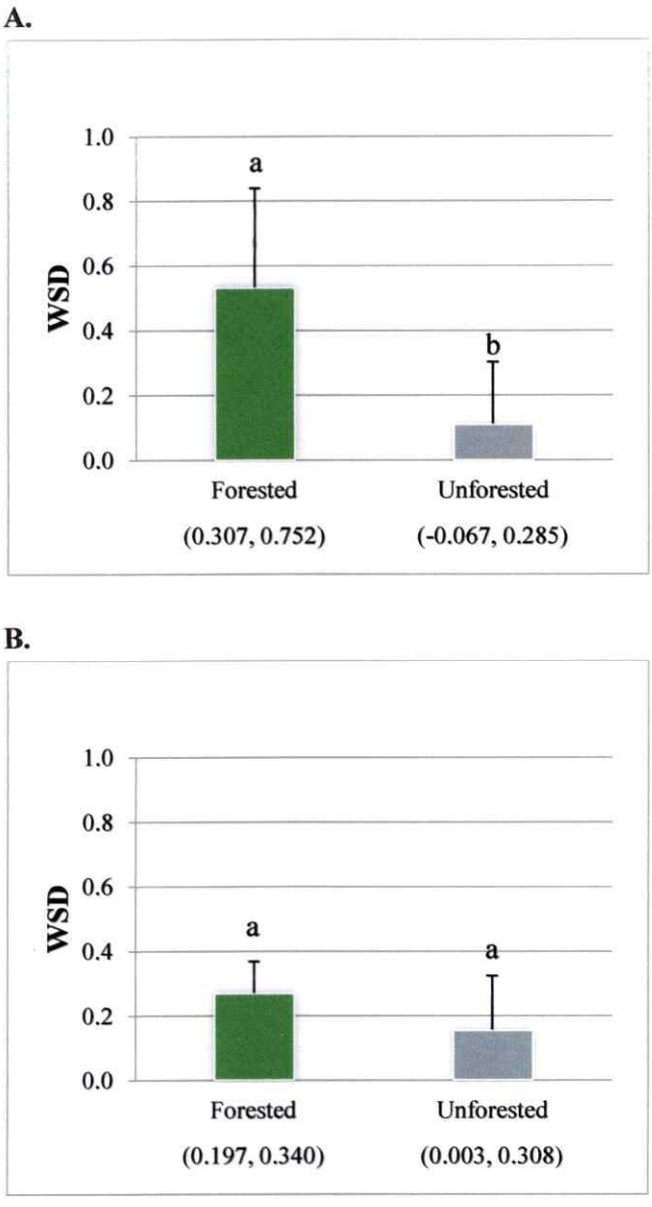


Figure 8. Weighted Shannon diversity (WSD) of the mycobionts (A) and the lichenized cyanobionts (B) per matrix type. Forested matrices: Coyhaique Forest 1, Coyhaique Forest 2, Karukinka Young-forest, Karukinka Mature-forest, Navarino Young-forest, Navarino Mature-forest; Un-forested matrices: Karukinka Grassland, Navarino Grassland, Deception Hillside. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

Results

To determine the effect of the latitude gradient of the sampling sites on the diversity of the symbionts, the sub-sites were grouped according to their geographic location (C, K, N and D). However, to eliminate the effect of the forest cover on these comparisons, only the sub-sites with similar matrix types were considered for the analyses along the latitudinal gradient. Given that in the un-forested matrices there was a single data per sampling site (Karukinka: KG; Navarino: NG; and Deception: DH) and, therefore, it was not possible to perform any statistical analyses on them, only the data from the forested matrices were analyzed (Coyhaique: CF and CF2; Karukinka: KY and NM; and Navarino: NY and NM). Figure 9 shows the WSD values calculated for the forested matrices of Coyhaique, Karukinka and Navarino in the case of the mycobionts (Figure 9A) and the lichenized cyanobionts (Figure 9B). Results showed that the mycobionts were most diverse in Coyhaique, and least diverse in Navarino. The cyanobionts, on the other hand, were more diverse in Karukinka than in Coyhaique and Navarino, where they presented similar WSD values. Only in the case of the mycobionts a linear relation was observed between diversity and latitude (R^2= 0.8806).

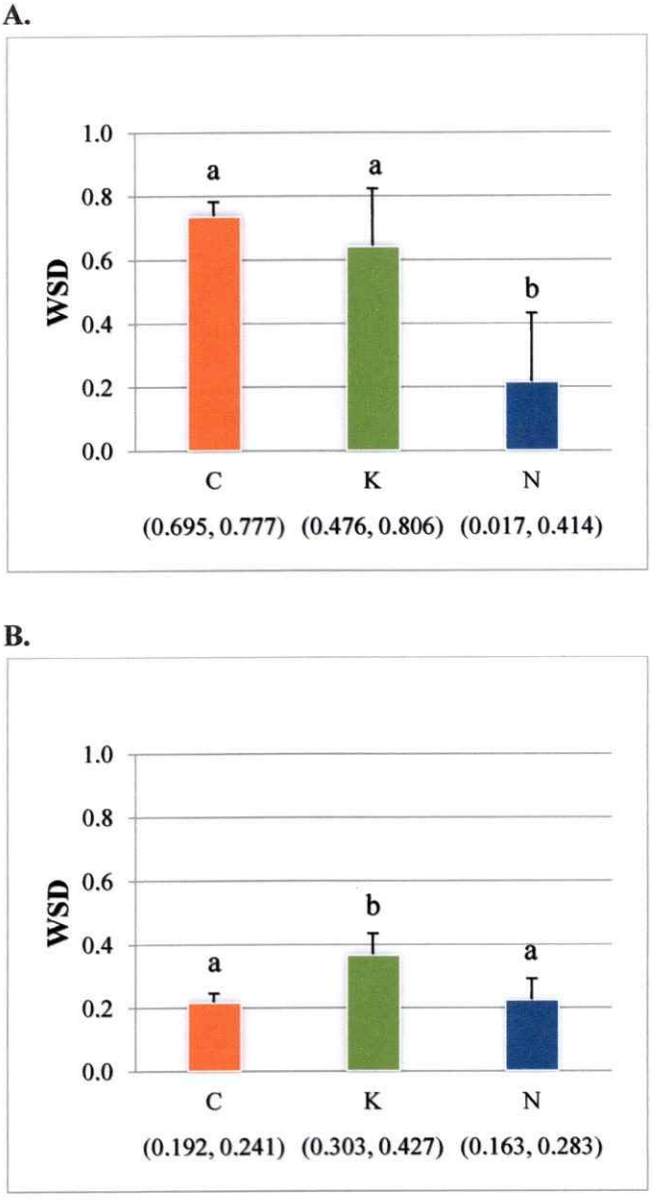


Figure 9. Weighted Shannon diversity (WSD) of the mycobionts (A) and the lichenized cyanobionts (B) per sampling site. C: Coyhaique, K: Karukinka, N: Navarino. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

3.3. Cyanobiont availability in the substrates

The abundance of the non-lichenized potential cyanobionts in the substrates (*(s)Cs*) was determined by cloning and sequencing the cyanobacterial SSU rDNA amplicons obtained from each of the 9 composite substrate samples (one per sub-site). Therefore, 9 libraries of 96 clones each were constructed, i.e., a total of 864 sequences were analyzed. Table 5 summarizes the information obtained from these clone libraries.

Table 5. Sequence analyses of the cyanobacterial clone libraries.

Sub-sites[a] →	**CF1**	**CF2**	**KY**	**KM**	**KG**	**NY**	**NM**	**NG**	**DH**	**average**
Number of clones	96	96	96	96	96	96	96	96	96	96
Nostoc abundance (%)[b]	37.5	28.1	32.3	24.0	4.2	42.7	49.0	55.2	44.8	35.3
Nostoc types	25	15	5	16	3	21	15	27	29	17.3
(s)C[c] abundance (%)[d]	10.4	15.6	19.8	4.2	0.0	14.6	25.0	20.8	15.6	14.0
(s)C types	1	3	2	3	0	3	1	3	1	1.9

[a]Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Karukinka Grassland (KG), Navarino Young-forest (NY), Navarino Mature-forest (NM), Navarino Grassland (NG), Deception Hillside (DH).
[bd]Abundance values (%) are presented with respect to the total number of clones analyzed in each library.
[c]*(s)Cs*: potential available cyanobionts in the substrate.

From the 864 clones analyzed, 67% corresponded to clones from forested matrices, and 33% to clones from un-forested matrices. In both matrix types, on average, 35.3% of the clones from each library corresponded to *Nostoc* sequences. However, this number varied considerably from one sub-site to the other, with a minimum of 4.2% of *Nostoc* clones in KG, and a maximum of 55.2% in NG. Within these *Nostoc* clones, there were

only considered as potential cyanobionts those that were a 100% identical (nucleotide identity) with the nucleotide sequence of the lichenized cyanobionts (*(l)Cs*) defined in section 3.1. (Figure 6, C1-C15). This number was variable, but in all cases low, representing on average a 14.0% of the total clones from each library, in both matrix types, with a mean value of ca. 2 different *(s)C* types per sub-site.

The abundance of the cyanobiont types available in the different sub-sites (*(a)Cs*), which includes the lichenized cyanobionts (*(l)Cs*) plus the ones obtained from the substrates (*(s)Cs*)) according to [3] (section 2.7.1.) is shown in Figure 10.

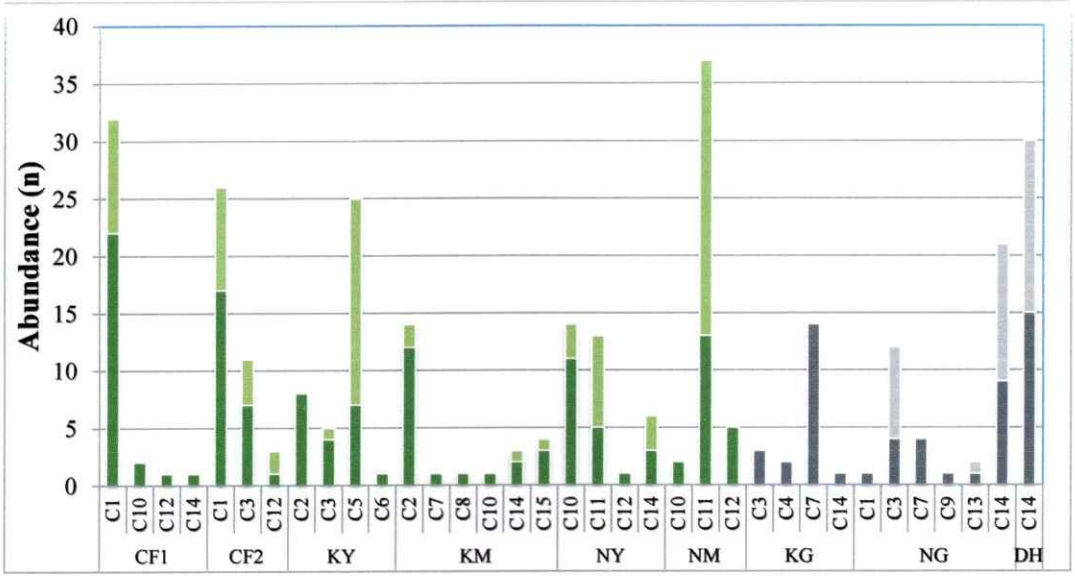


Figure 10. Cyanobiont availability per sub-site. C1-C15: cyanobiont OTUs. Dark inferior columns: lichenized cyanobionts, *(l)Cs*. Light superior columns: non-lichenized cyanobionts in the substrates, *(s)Cs*. Forested matrices (green tones): Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices (grey tones): Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).

As can be observed, from the 15 different cyanobiont OTUs defined in section 3.1., C4, C6, C7, C8 and C10 were undetected in the substrates. It can also be noticed that most cyanobionts were more abundant in the lichenized than in the non-lichenized state (dark columns vs light columns), with some exceptions like C5 (KY) or C11 (NY, NM), which were notoriously more abundant in the substrates than in the lichens. On the other hand, it is worth noticing that in each sub-site only the cyanobionts that were lichenized were also detected in the substrates, i.e., the cyanobionts that were not present in the lichens from a determined sub-site were also not detected in the respective substrates.

Figure 10 also shows that some cyanobionts were mainly exclusive to certain sub-sites, sampling sites or matrix types. Cyanobiont C1, for example, was mainly exclusive to Coyhaique forests, C2 was exclusively found in the forests of Karukinka, C11 was only present in the forested matrices of Navarino and cyanobionts C4, C5, C6, C10 and C13 were uniquely found in KG, KY, NG and NG, respectively. On the contrary, C14 was the most widely distributed cyanobiont, being present in 6 of the 9 sub-sites regardless of their forest cover or latitude. Furthermore, it was even present in DH, where it was the only cyanobiont available.

3.4. Available cyanobiont diversity

Once the availability of the potential cyanobiont types was determined in each sub-site, their diversity was calculated according to the Weighted Shannon Diversity Index (Table 6).

The most diverse sub-site regarding the available potential cyanobionts was KM followed by NY, and the least diverse sub-sites were DH and NM.

Table 6. Diversity values of the available potential cyanobionts.

	Sub-sites[a]	WSD[b]	WSDR[c]
Forested	CF1	0.150	VI
	CF2	0.270	IV
	KY	0.245	V
	KM	0.430	I
	NY	0.298	II
	NM	0.105	VIII
Un-forested	KG	0.133	VII
	NG	0.274	III
	DH	0.000	IX

[a]Forested matrices: Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices: Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).
[b]WSD: Weighted Shannon Diversity.
[cf]WSDR: Weighted Shannon Diversity Rank. I: most diverse; IX: least diverse.

When looking at the distribution of the available potential cyanobionts in these sub-sites (Figure 10) it can be seen that KM presented the highest richness of cyanobionts (n= 6) while NY presented an intermediate richness (n= 3). However, KM was dominated by one of these 6 cyanobionts (C2) whereas in the case of NY the 3 cyanobionts were evenly distributed, which partly explains its high diversity. In the case of the least diverse sub-sites, DH only presented a single cyanobiont type, which is the reason why its diversity value was zero. NM, on its part, despite having 3 different types of cyanobionts, was dominated by one of them (C11), which explain its low diversity.

When grouping the sub-sites according to their forest cover (Figure 11), results showed no significant differences between the diversity of the available cyanobionts per matrix type; whilst when grouping the forested matrices per sampling site in order to determine if changes in diversity were related with the latitudinal gradient of the sites (Figure 12), results showed no linear relation between diversity and latitude (R^2= 0.0029).

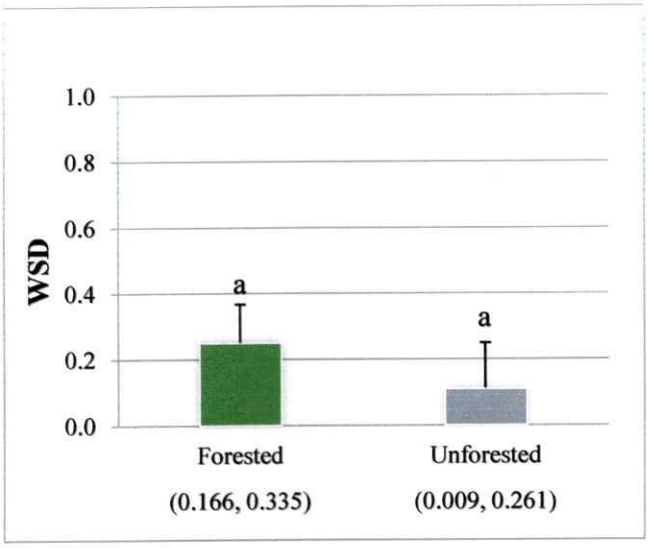


Figure 11. Weighted Shannon diversity (WSD) of the available potential cyanobionts per matrix type. Forested matrices: Coyhaique Forest 1, Coyhaique Forest 2, Karukinka Young-forest, Karukinka Mature-forest, Navarino Young-forest, Navarino Mature-forest; Un-forested matrices: Karukinka Grassland, Navarino Grassland, Deception Hillside. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

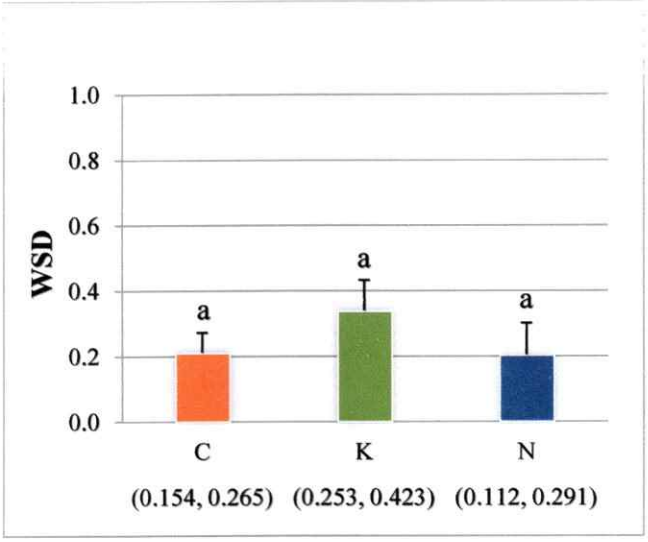


Figure 12. Weighted Shannon diversity (WSD) of the available cyanobionts per sampling site. C: Coyhaique, K: Karukinka, N: Navarino. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

3.5. Mycobiont specificity

Commonly, specificity is defined as the range of genetically compatible photobionts associated with a mycobiont which, according to Poulin *et al.* (2011), corresponds to the concept of *Basic Specificity*. Figure 14 shows the different pairs formed between the 8 mycobiont OTUs (M1-M8) and the cyanobiont OTUs they associated with (C1-C15).

As can be seen in Figure 13, mycobionts M3 and M7 were associated with only one cyanobiont, C8 and C14, respectively, which indicates a high specificity of these cyanobionts for their symbionts. Mycobionts M6 and M8 were both associated with 3 cyanobionts OTUs each, but the ones specific for M6 were more similar between them (had less nucleotide substitutions) than the ones for M8, suggesting that M6 was more specific in its association than M8. In the case of M4 and M5, both associated with 5

cyanobacterial OTUs, the ones associated with M4 were more similar between them than the ones associated with M5. On their part, mycobionts M2 and M1 associated with 4 and 6 cyanobiont OTUs respectively, the latter having the highest number of nucleotide substitutions between them. Finally, it can also be observed that many of the cyanobionts were associated with more than one mycobiont, being the case of C14 the most extreme, since it associated with 6 of the 8 mycobionts (M1, M2, M4, M5, M6 and M7).

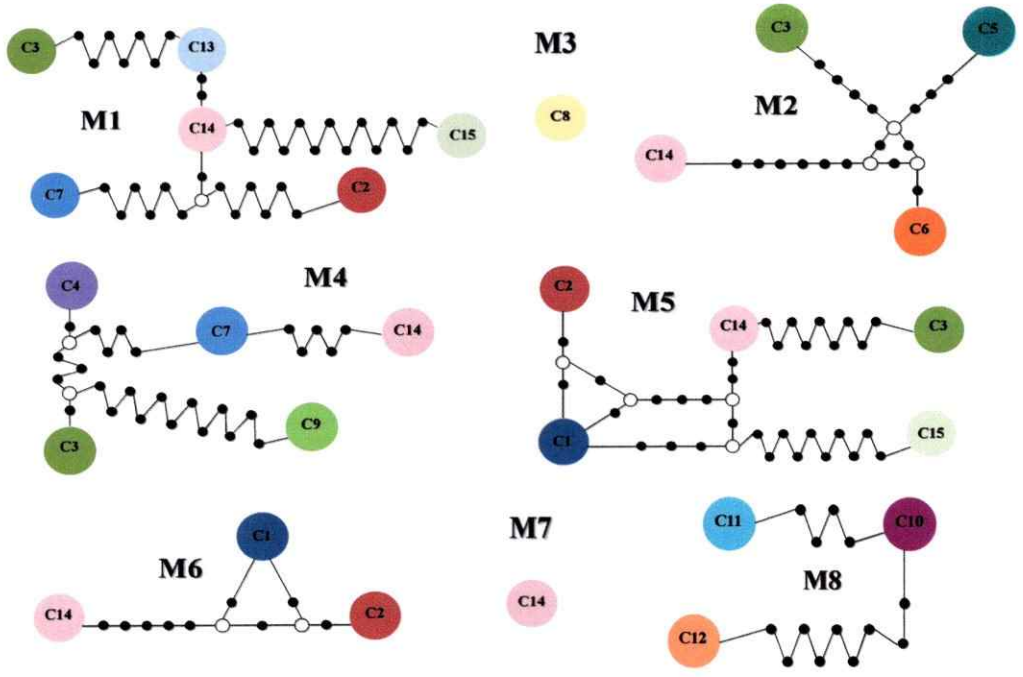


Figure 13. Symbiotic pairs. The 8 mycobionts (M1-M8) and their associated cyanobionts (C1-C15) are shown. Coloured circles represent the cyanobionts associated with each mycobiont. Black dots indicate the number of nucleotides that differ from each haplotype and the white ones the intermediate haplotypes which allow the network construction.

However, *Basic Specificity* does not consider the differences that exist among the symbiotic partners or the taxonomic (phylogenetic) distances that one mycobiont had to "overcome" to associate with different cyanobionts. For that reason, in the present study specificity was addressed under the concept of *Phylogenetic Specificity* (Poulin *et al.*, 2011), defined as the number of different cyanobiont partners (OTUs) with which the mycobionts associate, considering the genetic relatedness between these cyanobionts. Under that concept, a formula for calculating the *Local Phylogenetic Specificity* (LPSp) of each mycobiont was designed ([4]),

$$LPSp = \sum nc_i \qquad [4]$$

with $\sum nc_i$ being the total number of specific cyanobionts associated with a determined mycobiont, weighted by their relatedness according to [5];

$$nc_i = \left[\frac{1}{n} + \left(1 - \frac{1}{n}\right) \times \alpha_i\right] \qquad [5]$$

and n being the total number of discrete (not weighted) cyanobiont OTUs associated with each mycobiont. As described in [2] (section 2.7.1.), α_i corresponds to the correction factor that includes the genetic relatedness between the cyanobiont OTUs. The term "local" refers to the fact that "specificity" is a concept that should consider all the partners a mycobiont has been found associated with on a global scale, and this work only considers data from some localities in Southern Chile and Antarctica.

Table 7 indicates the LPSp and LPSpR values calculated for each mycobiont according to [4]. It can be observed that mycobiont M1 was the least specific mycobiont (LPSp= 2.281; LPSpR= VII), being associated with the highest number of cyanobiont types (C2, C3, C7, C13, C14 and C15), which had an average p-distance between them

of 0.256. It was followed by M5 (LPSp= 2.125; LPSpR= VI), that was associated with 5 different cyanobionts (C1, C2, C3, C14 and C15), with an average p-distance of 0.281. The next mycobionts, according to their specificity, were M4 (LPSp= 1.895; LPSpR= V), M8 (LPSp= 1.497; LPSpR= IV), M2 (LPSp= 1.445; LPSpR= III) and M6 (LPSp= 1.249; LPSpR= II), which were associated with 5, 3, 4 and 3 cyanobionts, respectively. Finally, since mycobionts M3 and M7 were associated with only one cyanobiont, (C8 and C14, respectively), they were assigned the highest specificity values.

Table 7. Mycobiont specificity and specificity rank.

Mycobiont OTU	Associated cyanobiont OTUs	Average α_i[a]	LPSp[b]	LPSpR[c]
M1	C2, C3, C7, C13, C14, C15	0.256	2.281	VII
M2	C3, C5, C6, C14	0.148	1.445	III
M3	C8	0.000	0.000	I
M4	C3, C4, C7, C9, C14	0.224	1.895	V
M5	C1, C2, C3, C14, C15	0.281	2.125	VI
M6	C1, C2, C14	0.124	1.249	II
M7	C14	0.000	0.000	I
M8	C10, C11, C12	0.249	1.497	IV

[a] α_i= correction factor (see [2], section 2.7.1.)
[b] Local Phylogenetic Specificity.
[c] Local Phylogenetic Specificity Rank. I: most specific; VII: least specific.

It is worth noticing the effect of the distance values in the calculus of specificity; for example, mycobiont M6 was more specific than M8, despite the fact that they were both

associated with the same number of cyanobionts (see explanation for Figure 14). Moreover, mycobiont M2 was also more specific than M8, even though it was associated with less cyanobiont types (3 and 4, respectively).

3.6. Ecological success

The ecological success of the symbiotic pairs reflects the environmental fitness of the holobionts and, therefore, was defined in this study as the abundance of the associated compatible symbiotic pairs in each sub-site (Figure 14). It can be observed that in the forested matrices (green columns) there were 21 different symbiotic pairs: M1-C2, M1-C3, M1-C15, M2-C3, M2-C5, M2-C6, M3-C8, M4-C3, M4-C7, M5-C1, M5-C2, M5-C3, M5-C14, M5-C15, M6-C1, M6-C2, M6-C14, M7-C14, M8-C10, M8-C11 and M8-C12. From these, the most ecologically successful was M5-C1, with 21 representatives, followed by M6-C1 and M8-C11, with 18 representatives each.

On the other hand, in the un-forested matrices (grey columns) there were 10 different symbiotic pairs: M1-C7, M1-C13, M1-C14, M2-C14, M4-C3, M4-C4, M4-C7, M4-C9, M4-C14 and M5-C1. It is worth noticing that symbiotic pair M2-C14, the most successful holobiont in the un-forested matrices with 15 representatives, was only present in Deception Hillside (DH), which could be considered as the most extreme environment from this study.

Only 3 symbiotic pairs were both in the forested and the un-forested matrices: M4-C3, M4-C7 and M5-C1. From them, M4-C3 was similarly successful in both types of matrices, while M4-C7 was clearly more successful in the un-forested matrices and M5-C1 presented the opposite behaviour, being more successful in the forested ones.

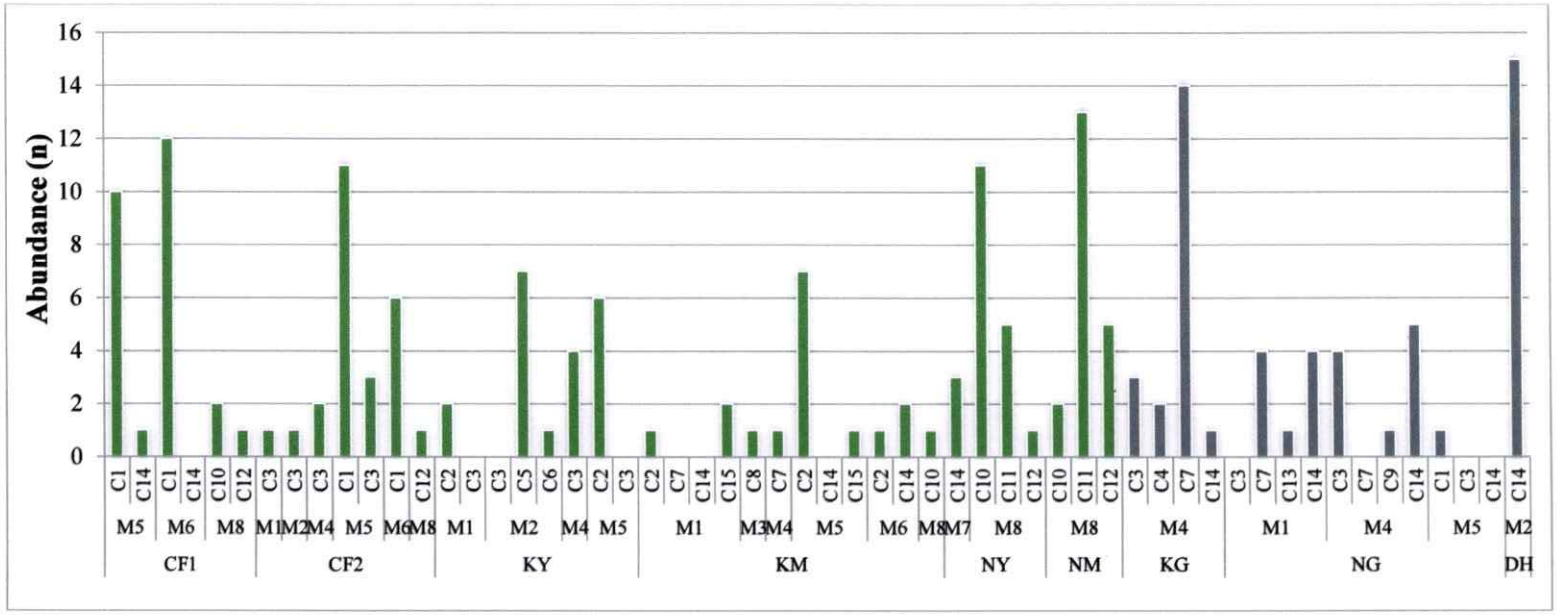


Figure 14. Ecological success of the symbiotic pairs per sub-site. M1-M8: Mycobiont OTUs; C1-C15: cyanobiont OTUs. Forested matrices (green): Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices (grey): Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).

Results

The average abundance or ecological success of the symbiotic pairs in each sub-site was calculated (Table 8) and results showed that on average the sub-site in which the symbiotic pairs present were most successful was DH, while the one in which they were least successful was KM.

Table 8. Average Ecological Success of the symbiotic pairs per sub-site.

	Sub-sites[a]	Average ES[b]	ESR[c]
Forested	CF1	3.63 ± 2.50	V
	CF2	3.00 ± 2.76	VI
	KY	2.67 ± 1.25	VII
	KM	1.32 ± 0.70	IX
	NY	4.33 ± 1.89	IV
	NM	6.67 ± 0.00	II
Un-forested	KG	5.00 ± 0.00	III
	NG	1.69 ± 1.19	VIII
	DH	15.00 ± 0.00	I

[a]Forested matrices: Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices: Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).
[b]ES: Ecological Success.
[c]ESR: Ecological Success Rank. I: most successful; IX: least successful.

To determine the effect of the forest cover on the ecological success, the ES values calculated for the different symbiotic pairs present in each of the sub-sites were grouped according to the forest cover of that sub-site, as presented in Figure 15. It can be

observed that the type of matrix does not influence the average ecological success of the symbiotic pairs.

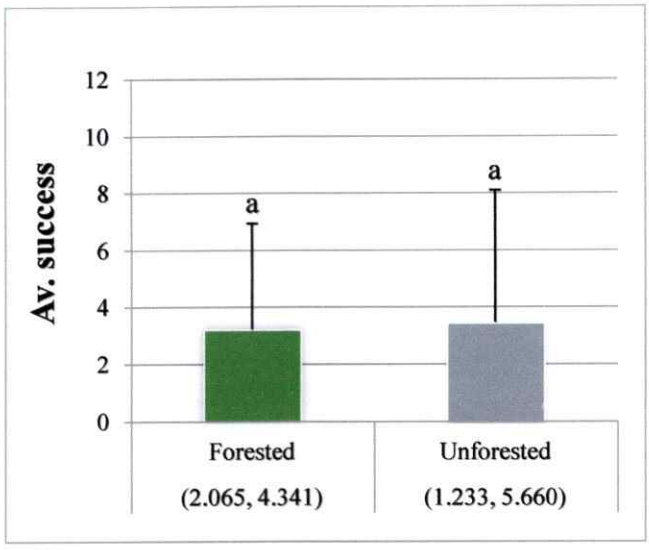


Figure 15. Average Ecological Success (ES) of the symbiotic pairs per matrix type. Forested matrices: Coyhaique Forest 1, Coyhaique Forest 2, Karukinka Young-forest, Karukinka Mature-forest, Navarino Young-forest, Navarino Mature-forest; Un-forested matrices: Karukinka Grassland, Navarino Grassland, Deception Hillside. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

Finally, to determine the effect of the latitude on the ecological success of the symbiotic pairs, the ES values calculated for the different symbiotic pairs present in each of the sub-sites were grouped according to their geographical origin (sampling sites), as shown in Figure 16. No relation was observed between the latitudinal gradient of the sampling sites and the average ecological success of the symbiotic pairs.

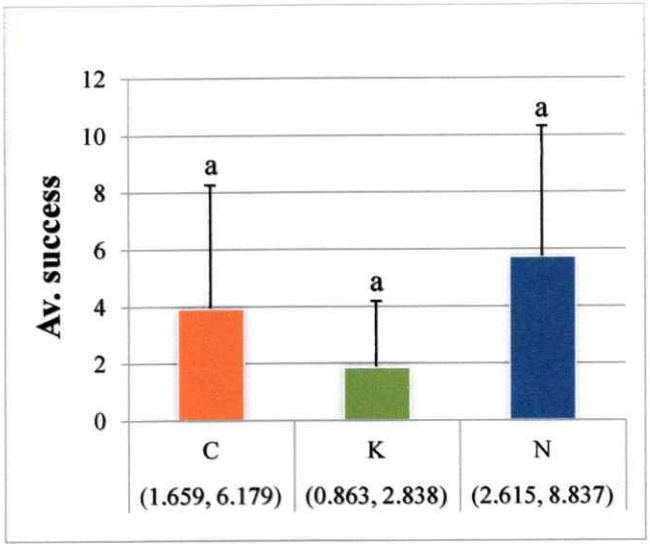


Figure 16. Average Ecological Success (ES) of the symbiotic pairs per sampling site. C: Coyhaique, K: Karukinka, N: Navarino. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

3.7. Mycobiont selectivity

Selectivity is theoretically defined as the preferential association of the mycobionts with some of their specific cyanobionts. Given that there are no indices that can quantitatively determine selectivity in symbiotic associations, the first part of this objective consisted in designing an index for calculating the selectivity of each mycobiont for its respective cyanobionts. This index should consider among its variables the environmental abundance of the potential cyanobionts, that is, their availability; the different cyanobiont OTUs each mycobiont was associated with, namely, their specificity; and the number of cyanobiont OTUs effectively used by each mycobiont, i.e., the ecological success of the symbiotic pairs. For that purpose, the Eligibility Index E_i^* ([6]), designed by Vanderploeg & Scavia (1979) for determining the preference of

animals for certain types of food, was chosen from the revision made by Lechowicz (1982) since it meets certain characteristics that are desirable in any index of electivity, such as being a random symmetric model whose values move within a range of possible results, among others (Lechowicz, 1982). This index considers: *i*) the total number of food types that could be selected by an animal (n) and *ii*) a selectivity coefficient (W_i) ([7]), which, on its part, relates: *i*) the frequency of food types actually selected (r_i), *ii*) the frequency of food types available in the environment (p_i).

$$E_i^* = \frac{W_i - \frac{1}{n}}{W_i + \frac{1}{n}} \quad [6]$$

$$W_i = \frac{\left(\frac{r_i}{p_i}\right)}{\sum\left(\frac{r_i}{p_i}\right)} \quad [7]$$

The modified index proposed in the present work was named Selectivity index (Se_i) and was adapted from [6], according to [8]

$$Se_i = \frac{W_i^* - \frac{nc_i}{\sum nc_i}}{W_i^* + \frac{nc_i}{\sum nc_i}} \quad [8]$$

with W_i^* ([9]) being the modified selectivity coefficient, r_i the frequency of the different cyanobiont OTUs selected, p_i the frequency of the available cyanobionts OTUs in the environment and nc_i the total number of cyanobiont OTUs weighted by their genetic relatedness (see [5], section 3.5.).

$$W_i^* = \frac{nc_i\left(\frac{r_i}{p_i}\right)}{\sum nc_i\left(\frac{r_i}{p_i}\right)} \quad [9]$$

Both, the original and the new index, adopt a value of cero for a random selection, and a possible range of plus one (+1) to minus one (-1) depending if the item (food type or cyanobiont OTU) is considered preferred or avoided, respectively (Lechowicz 1982).

Since the number of the observed symbiotic pairs was different from the number of the expected ones (G-test: observed vs. expected; $p<0.0001$), then the associations were not random and therefore, their selectivity was calculated.

Figure 17 shows the selectivity values calculated using the Se_i index for the symbiotic pairs present in the different sub-sites.

As above mentioned, the different values ranged between +1 and -1, adopting a value of 0 if the association is considered to be random, as was the case of all the symbiotic pairs formed by M4 in KG, because its specific cyanobiont OTUs were selected in the same proportion in which they were available. If there was only one specific cyanobiont OTUs available for a determined mycobiont (Figure 17, black stars), selectivity was not calculated because it was considered that there was nothing to choose from. From these values the average selectivity of the mycobionts present in each sub-site was determined, as summarized in Table 9.

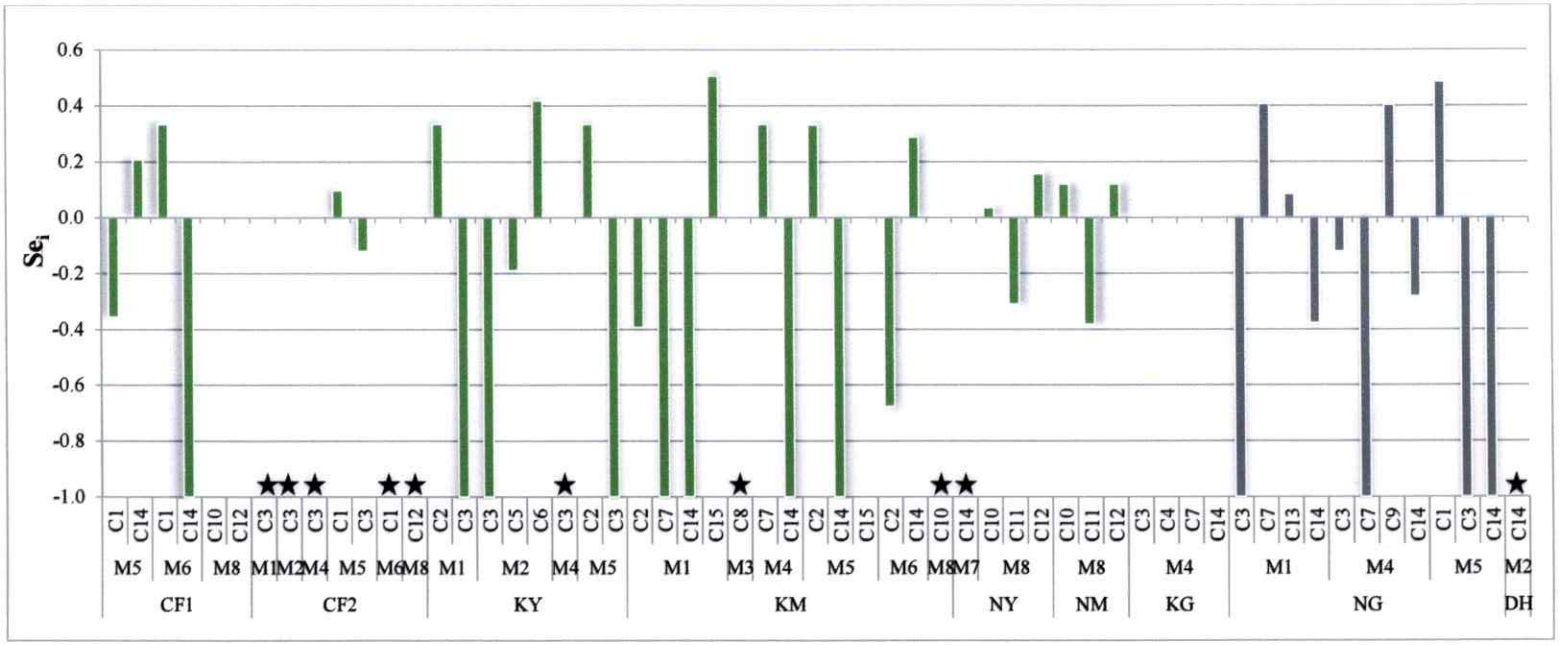


Figure 17. Selectivity of the mycobionts per sub-site. M1-M8: Mycobiont OTUs; C1-C15: cyanobiont OTUs. Forested matrices (green): Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices (grey): Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH). Black stars indicate that the index was not calculated for that symbiotic pair. The possible results for the index move between a range of plus one (+1) to minus one (-1) depending if the cyanobiont is considered preferred or avoided, respectively.

Table 9. Average selectivity of the mycobionts per sub-site.

	Sub-sites[a]	Av.Se_i[b]	Se_iR[c]
Forested	CF1	0.316 ± 0.335	IV
	CF2	0.018 ± 0.000	VII
	KY	0.467 ± 0.076	II
	KM	0.386 ± 0.137	III
	NY	0.084 ± 0.000	VI
	NM	0.208 ± 0.000	V
Un-forested	KG	0.000 ± 0.000	VIII
	NG	0.583 ± 0.213	I
	DH	ND[d]	ND

[a] Forested matrices: Coyhaique Forest 1 (CF1), Coyhaique Forest 2 (CF2), Karukinka Young-forest (KY), Karukinka Mature-forest (KM), Navarino Young-forest (NY), Navarino Mature-forest (NM); Un-forested matrices: Karukinka Grassland (KG), Navarino Grassland (NG), Deception Hillside (DH).
[b] Av.Se_i: Average Selectivity index.
[c] Se_iR: Selectivity Rank. I: most selective; VIII: least selective.
[d] ND: not determined

As shown in Table 9, the sub-site where the mycobionts were mostly selective was NG (Se_iR= I), followed by KY (Se_iR= II). On the other hand, the sub-site where the mycobionts were least selective was KG (Se_iR= VIII), because the cyanobionts selected in that sub-site were in the same proportion as their availability (random association).

To determine if selectivity was related to the type of matrix, the selectivities of the mycobionts in each sub-site (Table 9) were grouped according to the forest cover, and the average mycobiont selectivity per matrix type was calculated (Figure 18). There was no relation between the type of matrix and the selectivity of the mycobionts.

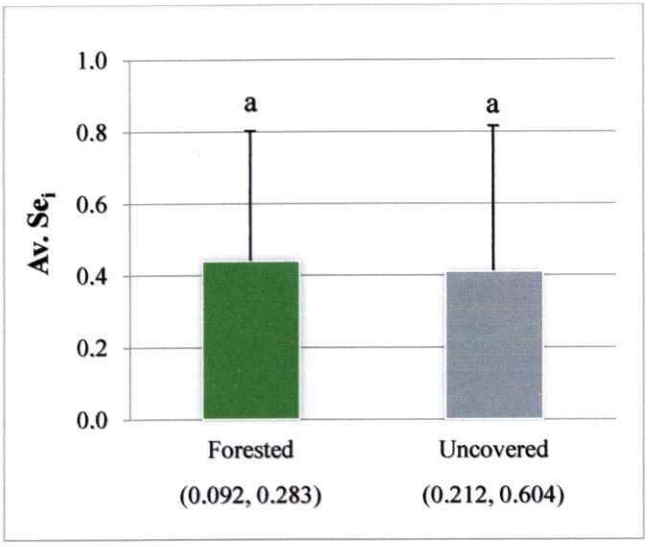


Figure 18. Average Se_i of the mycobionts per matrix type. Forested matrices: Coyhaique Forest 1, Coyhaique Forest 2, Karukinka Young-forest, Karukinka Mature-forest, Navarino Young-forest, Navarino Mature-forest; Un-forested matrices: Karukinka Grassland, Navarino Grassland, Deception Hillside. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

The average mycobiont selectivity was also calculated per geographic origin (sampling site) for the forested matrices, in order to determine if there was a relation between the latitudinal gradient of the sites and the selectivity values observed. As shown in the Figure 19, there was no correlation between selectivity and the latitudinal gradient of the sites (R^2= 0.0047).

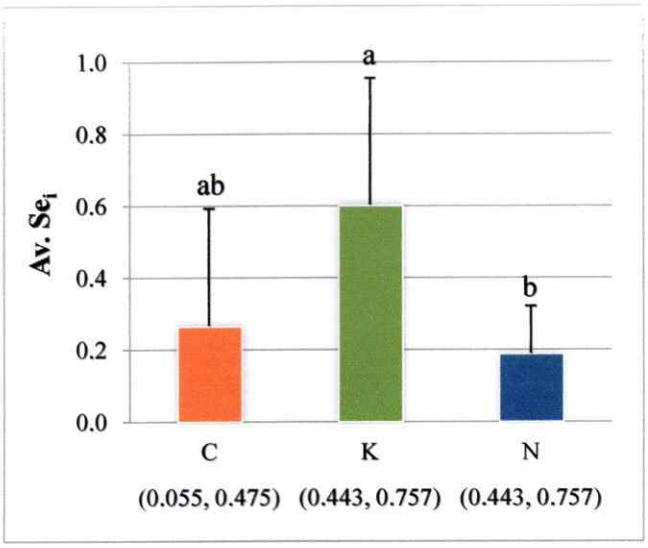


Figure 19. Average Se_i of the mycobionts per sampling site. C: Coyhaique, K: Karukinka, N: Navarino. Numbers in parenthesis correspond to the extreme values of the confidence intervals (10,000 random re-samplings). Error bars correspond to standard deviations. Different letters over error bars indicate statistically significant differences.

Finally, selectivity was also calculated for each mycobiont considering all the sites where they were present, and was compared with the average ecological success of the symbiotic pairs they formed. As shown in Table 10, the most selective mycobionts, M1, M2 and M6 (Se_iR= II, III and I, respectively), were the least successful ones (ESR= VI, V and IV, respectively), while the least selective mycobionts, M4, M5 and M8 (Se_iR= V, IV and VI, respectively) were the most successful ones (ESR= III, II and I, respectively), i.e., there was an inverse relation between the selectivity of the mycobionts and their ecological success.

Table 10. Average Selectivity and Ecological Success per mycobiont.

Ms[a]	Molecular Identity	Total Pairs formed (n)	Av. Se_i[b]	Se_iR[c]	Av. ES[d]	ESR[e]
M1	*P. ponojensis*	18	0.425 ± 0.290	II	3.00 ± 2.00	VI
M2	*P. extenuata*	24	0.412 ± 0.317	III	6.00 ± 6.63	V
M3	*Peltigera* sp.	1	ND[f]	ND	1.00 ± 0.00	VII
M4	*P. rufescens*	37	0.288 ± 0.266	V	7.40 ± 6.35	III
M5	Lin *P. canina*	40	0.385 ± 0.328	IV	8.00 ± 9.27	II
M6	*P. frigida*	*21*	0.491 ± 0.086	I	7.00 ± 9.54	IV
M7	Lin *P. neckeri*	3	ND	ND	3.00 ± 0.00	VI
M8	Lin *P. hymenina*	42	0.169 ± 0.114	VI	14.00 ± 5.29	I

[a]*Ms*: mycobionts.
[b]Av.Se_i: Average Selectivity index.
[c]Se_iR: Selectivity Rank. I: most selective; VI: least selective.
[d]ES: Ecological Success.
[e]ESR: Ecological Success Rank. I: most successful; VII: least successful.
[f]ND: not determined

DISCUSSION

The ecological and genetic factors determining the development of a successful lichen symbiosis are still poorly understood. Based on the conceptual model proposed by Yahr *et al.* (2006), photobiont availability in a given locality determines which lichens can occur locally. From the perspective of the mycobiont, specificity describes the phylogenetic diversity of the possible photobionts. Only those mycobionts that can associate with the locally present photobionts have a chance of establishing successful populations. Finally, selectivity measures the frequency of association with genetically different photobionts in a locality (Rambold *et al.*, 1998; Yahr *et al.*, 2004; Fernández-Mendoza *et al.*, 2011).

In this thesis, the diversity of the symbiotic pairs in *Peltigera* cyanolichens was calculated in different sites and ecological contexts and, from the operational taxonomic units (OTUs) identified, the ecological factors proposed in the lichenization model were determined.

4.1. Symbiont diversity

For determining the mycobiont and cyanobiont diversity in the *Peltigera* lichen samples, OTUs were defined as those symbionts that were 100% identical in their nucleotide sequence, which gave a total of 8 mycobiont OTUs and 15 cyanobiont OTUs. In the case of the mycobionts, this classification was corroborated with a morphological revision of the samples considering characteristics such as the shape of the thallus, shape

and size of the lobes, presence or absence of reproductive structures, type of reproductive structures when present, etc. In all cases, similar characteristics were shared among the established groups and different between them, giving support to the molecular classification.

A phylogenetic analysis of the mycobionts including sequences from Europe, North America, Asia, Australia and a few from South America (Brazil, Argentina, Venezuela and Ecuador), all previously described in key treatments (Miadlikowska & Lutzoni, 2000; Goffinet *et al.*, 2003; Miadlikowska *et al.*, 2003), showed that most of our mycobionts (6/8) were part of the infra-generic section *Peltigera,* while the other 2 belonged to sections *Horizontales* and *Polydactylon*, respectively. Four OTUs could be named with a high degree of confidence, being supported on short branches within clusters of named accessions (M1, M2, M4 and M6).

Previous reports about the diversity of *Peltigera* in the studied regions are scarce. Quilhot *et al.* (2012) and Martínez *et al.* (2003) described the lichen biota from Aysén Region, which includes the National Reserve of Coyhaique, and found 14 *Peltigera* species; however, neither of these reports included *P. ponojensis,* which in this work was represented by 18 specimens. At Karukinka, the only published reports of *Peltigera* correspond to a previous baseline study conducted in different areas of the Park (Arroyo *et al.*, 1996), our previous studies (Ramírez-Fernández *et al.*, 2013; Ramírez-Fernández *et al.*, 2014) including the Karukinka specimens from the present work, and the revision of Martínez *et al.* (2003) about the global distribution of *Peltigera* lichens. For the other 2 sites, previous reports only mention a small number of species including *P. rufescens* in the case of Navarino (Méndez *et al.*, 2012) and *P. didactyla* in Deception Island

(Øvstedal & Lewis-Smith, 2001; ASPA 140, 2005; Spielman & Pereira, 2012). Interestingly, in our analysis, none of the mycobionts were supported as related to *P. ulcerata* or *P. laciniata*, even though the first has been reported in Aysén (Quilhot *et al.*, 2012) and the second is a species restricted to Central and South America (Martínez *et al.*, 2003). It is possible that these species were present at the sites, but rare enough to remain un-sampled.

The distribution of the lineages documented by this work fit with some biogeographic expectations, but also offer some surprises. For example, *P. frigida* (related to M6) has been exclusively described in the southernmost part of South America (Miadlikowska & Lutzoni, 2000; Martínez *et al.*, 2003); however, species from *P. neckeri* lineage (related to M7) and *P. hymenina* lineage (related to M8) have scarce reports in South America (Quilhot *et al.*, 2012; Martínez *et al.*, 2003), being defined commonly as circumpolar (Martínez *et al.*, 2003). Furthermore, to our knowledge, there are no reports of *P. ponojensis* (related to M1) in South America, except our previous works from Karukinka (Ramírez-Fernández *et al.*, 2013; Ramírez-Fernández *et al.*, 2014). One specimen, M3, was not related to any published sequence from the data base, which indicated that it could correspond to a new undescribed species. Given that *Peltigera* has been poorly explored in the sampled regions it is possible that this lichen corresponds to a novel South American species, showing how DNA-based identification, despite its great potential to accurately identify a high percentage of specimens to the correct species, is limited by the availability of accurate baseline taxonomic data (Nilsson *et al.*, 2006; Begerow *et al.*, 2010; Orock *et al.*, 2012). Moreover, apart from not being accurately

identified, this mycobiont probably corresponds to a rare species since from the nearly 200 lichens sampled it was only represented by a single specimen.

Consistent with previous studies, tripartite *Peltigera* species were not detected at the sampled locations, regardless of their apparent abundance in studies from the northern hemisphere (O'Brien *et al.*, 2009; Wei *et al.*, 2009).

Despite many efforts to amplify the nuclear ribosomal internal transcribed spacer (ITS), which has been proposed as the universal DNA barcode marker for Fungi (Schoch *et al.*, 2012), in many cases it was not possible to obtain the high quality sequences necessary for a phylogenetic analysis. In the genus *Peltigera*, mononucleotide runs are common in the ITS (Miadlikowska *et al.*, 2003) and are known to cause problems in Sanger sequencing (Kircher & Kelso, 2010). For that reason the LSU rDNA was used instead, even though it is not the highest resolution marker available for fungi and, therefore, it is worth considering the results in the possible light of further resolution of mycobionts.

Although we are relatively confident about the identity of half of our recognized specimens due to their short branch lengths and strong statistical support in the phylogenetic analysis, identification using comparisons with extant sequenced vouchers leaves open two possibilities that must be considered. First, sequenced voucher specimens could be misidentified, which is not an uncommon problem, particularly for fungal accessions in GenBank (Bridge *et al.*, 2003; Nilsson *et al.*, 2006). Furthermore, for the relatively poorly explored South American continent (Martínez *et al.*, 2003), there is a good chance that unrecognized diversity exists, and that therefore no identified or sequenced vouchers will exist for some of the lineages actually present in Chile.

Further work is required to fully explore the morphological and genetic diversity present, using further surveys in addition to higher resolution markers.

With respect to the cyanobionts, all symbiotic *Nostoc* strains determined in this study fell within the *Peltigera* guild reported by Rikkinen *et al.* (2002) and within the *Nostoc* II clade reported by O'Brien *et al.* (2005). According to these authors, all *Nostoc* strains that are symbiotically associated with *Peltigera* belong to this group, along with other symbiotic and free living strains, and it is possible that they all comprise one single species. The *Nostoc* sequences from the present work did not group according to the latitudinal gradient of the sub-sites, and did not even separate from the northern hemisphere ones obtained from the data base, contrary to what was observed by Iglesias Fernández (2009), who concluded that the site where lichens grow seems to determine what strain of *Nostoc* appears in some *Peltigera* symbiosis from Iceland. Nonetheless, our results seem to agree with the ones published by Stenroos *et al.* (2006) whose *Nostoc* clades did not correlate with the geographic origin of the lichens, indicating that many *Nostoc* taxa are very widely distributed.

In general, the sequences related to the cyanobionts of the present study were from specimens collected in North America and Europe, with the exceptions of C1 and C12, which are most similar to cyanobionts previously described from South America, particularly from Argentina (Kaasalainen *et al.*, 2012), and C8, which was one of the cyanobionts that did not closely associate with any known *Nostoc* sequences. This is interesting because this cyanobiont was exclusively associated with M3, the mycobiont that did not relate with any of the known *Peltigera*. None of the cyanobacterial sequences of the present study was directly related with sequences from Oceania, also

from *Nostoc* clade II, despite the recognized floristic relationship existing, for example, between New Zealand and Southern Chile (Ezcurra *et al.*, 2008). Finally, the only cyanobiont present in Antarctica, C14, was closely related to a *Peltigera* cyanobiont from Scotland (Kaasalainen *et al.*, 2012), a humid and temperate region with conditions very distant from those present in Antarctica. However, the fact that it was also present in lichens from Coyhaique, Karukinka and Navarino suggests this is a versatile strain, capable of adapting to different ecological conditions.

Symbiont diversity was determined according to the Weighted Shannon's Diversity Index (H_w) (Casquilho *et al.*, 1997) which considers the richness and the frequency of the symbionts in a determined site, along with their genetic relatedness. This last factor is very important since it "corrects" the diversity values obtained by the normal Shannon index, considering that if the symbionts of a determined site are more related than the ones from another, then their diversity should be lower.

As shown in section 3.2., the mycobionts were more diverse in the forested matrices than in the un-forested ones. Given that lichens are poikilohydric, i.e., are dependent upon direct inputs of water-vapor, dew and precipitation for photosynthesis and the maintenance of growth (Nash, 2008), a higher diversity in more humid, protected environments such as forests is not surprising. This also agrees with works reporting that forest degradation may influence the diversity of epiphytic (e.g. Ellis *et al.*, 2007; Fritz *et al.*, 2008; Johansson 2008; Belinchón *et al.*, 2009; Aragón *et al.*, 2010; Otálora *et al.*, 2011; Király *et al.*, 2013) and terricolous (Scutari *et al.*, 2004; Motiejûnaitë & Faùtynowicz, 2005; St. Clair *et al.*, 2007; Rai *et al.*, 2012) lichen communities. Our previous work on the diversity of terricolous *Peltigera* in different environmental

contexts of Karukinka (Ramírez-Fernández *et al.*, 2013) also showed that it was higher in undisturbed forested environments than in degraded un-forested ones, tendency that was maintained with the inclusion of more sampling sites, such as Coyhaique, Navarino and Deception.

In the case of the cyanobionts, the average diversity was similar in the forested and un-forested environments (Figure 8B), and individually the most diverse sites were KM (Karukinka Mature-forest) and NG (Navarino Grassland), a forested and an un-forested one, respectively. This is very interesting considering that, in contrast, NG (Navarino Grassland) was one of the least diverse sites for the mycobionts. Concerning this, some authors have determined that while the distribution of some symbiotic *Nostoc* genotypes are correlated with mycobiont taxonomy at the species or genus level, the distribution patterns of others are best explained by habitat ecology (Elvebakk *et al.*, 2008). Also, other factors such as specificity and selectivity are known to determine the lichenized cyanobionts (Rikkinen *et al.*, 2002; O'Brien *et al.*, 2005; Yahr *et al.*, 2006), so higher mycobiont diversity does not necessarily imply higher cyanobiont diversity.

Finally, in order to find out if there was a relation between the diversity of the symbionts and the latitudinal gradient of the sampling sites, the diversity values of the sub-sites with similar forest cover (forested matrices) were grouped according to their geographic origin. The linear decrease tendency observed in the diversity of the mycobionts with the increase of the latitude (Figure 9A) agrees with the general idea that there is a latitudinal biodiversity gradient (LBG) for which diversity reaches its minimum at higher latitudes (Willig *et al.*, 2003; Dowle *et al.*, 2013). In the case of the cyanobionts there was no linear relation between the latitude of the sampling sites and

their diversity (Figure 9B), agreeing with reports that have found latitudinal diversity gradients to be absent or weak in unicellular organisms, attributing this to their high abundance and dispersal capabilities and suggesting that bacteria, the smallest and most abundant organisms, should exhibit no latitudinal patterns of diversity (Fenchel & Finlay, 2004; Green & Bohannan, 2006; Fierer & Jackson, 2006; De Bie *et al.*, 2012). In any case, it must be kept in mind that most of the LBG theories are based on studies performed on free living (non-symbiotic) organisms (Fenchel & Finlay, 2004) and that the distribution patterns of symbionts might be limited to the distribution and availability of their respective hosts. Therefore, factors such as specificity and selectivity might have a higher impact on their distribution patterns (Rikkinen *et al.*, 2002; O'Brien *et al.*, 2005; Yahr *et al.*, 2006).

Even though Deception was not included in the latitudinal comparisons, it is interesting to observe that the diversity of both symbionts was null at that site, that is, there was only one type of each symbiont. Concerning this, it has been observed that green-algal lichens are the most successful species under such extreme conditions as they do not depend on the presence of liquid water for reactivation from their dry inactive state, in contrast to cyanobacterial lichens (Ruprecht *et al.*, 2012), which is probably why they are less frequent or even completely absent in the Antarctic cold deserts (Lange *et al,*. 1986; Kappen, 2000). On the other hand, Domaschke *et al.* (2012) compared the genetic diversity of Antarctic populations of the widespread chlorolichen *Cetraria aculeata* with that of populations from other continents, and reported that mycobiont diversity was highest in Arctic populations, while photobiont diversity decreased significantly towards the Antarctic but less markedly towards the Arctic. The

authors concluded that ecological factors play a minor role in determining the diversity of Antarctic photobiont populations and that the reduced genetic diversity in the Antarctic would be most likely due to founder effects during long-distance colonization.

4.2. Cyanobiont availability

Several strategies have been proposed that might allow lichen-forming fungi to reconstitute a lichen symbiosis in nature from one generation to the next. These include extracting photobiont cells from the thalli of other lichens (Friedl, 1987; Ott, 1987) or from the asexual propagules of other species (Rikkinen, 2003); and persistence by forming temporary associations with incompatible photobionts (Gassmann & Ott, 2000) or temporarily in a free-living state (Etges & Ott, 2001) until a compatible photobiont is encountered. For that reason, the available cyanobionts (*(a)Cs*) were defined as [3], i.e., the sum between *i*) the non-lichenized potential cyanobionts present in the substrates, *(s)Cs,* and *ii*) the lichenized cyanobionts, *(l)Cs*. On that subject, most studies have compared the photobionts of each lichen species with the pool of photobiont genotypes represented by co-occurring species at the same locations (Yahr *et al.*, 2004; Fedrowitz *et al.*, 2011; Lindblon & Sochting, 2013), addressing indirectly the photobiont availability. To our knowledge, only one study has assessed the availability of the potential photobionts, not from the substrate but from the surface of the chlorolichen *Protoparmeliopsis muralis* (Muggia *et al.*, 2013), concluding that lichen surfaces represent a potential temporary niche for free-living stages of lichen photobionts, which could facilitate the establishment of further lichens in the proximal area. Even though some studies about the lichen microbiome have been reported recently, none of them

analyzed the availability of the potential cyanobionts, either excluding them from the PCR amplifications using specific primers or from the posterior analyses (e.g. Hodkinson *et al.*, 2012; Printzen *et al.*, 2012; Grube *et al.*, 2014; Hodkinson *et al.*, 2014).

In the present study, the availability of the non-lichenized potential cyanobionts in the substrates was addressed through the construction of 9 clone libraries, each corresponding to one of the 9 sub-sites from where the samples were taken (Table 1). Since many samples were collected in these sub-sites, composite samples (1 per sub-site) were created in order to increase representativeness and reduce spatial heterogeneity. Both in the forested as in the un-forested matrices, around 35% of the clones obtained corresponded to *Nostoc* sequences, with a minimum abundance of *Nostoc* sp. clones in KG (Karukinka Grassland) and a maximum in NG (Navarino Grassland), both un-forested matrices. Although it has been suggested that availability is a factor directly influenced by the environmental conditions (Wirtz *et al.*, 2003; Yahr *et al.*, 2006), both sites possess similar ecological characteristics but very different *Nostoc* abundances. KG was also the sub-site with the lowest richness (different types) of *Nostoc* clones, while again NG and also DH (Deception hillside) presented the highest richness of *Nostoc* (Table 5). Given that DH is an extreme Antarctic environment, it is interesting to observe high richness and abundance levels of *Nostoc* strains in that site. Nevertheless, this agrees with studies reporting a high diversity of *Nostoc* strains in the Antarctic region, both free living and symbiotically associated with lichen forming fungi or bryophytes (Broady, 1996; Cary *et al.*, 2010; Micheli *et al.*, 2014), which is explained by their physiological versatility and ample ecological tolerance, allowing them to

compete successfully with other organisms in aquatic and terrestrial environments (Micheli *et al.*, 2014). Whatever the case, richness values were not necessarily correlated with abundance data, as can be clearly observed in the case of KY (Karukinka Young-forest), which presented a relatively high abundance of *Nostoc* clones but had one of the lowest *Nostoc* richness values (Table 5).

From the *Nostoc* sp. clones determined in the substrates, were considered as potential available cyanobionts those whose nucleotide sequence was 100% identical with the sequence of any of the cyanobionts determined in the lichen samples (C1-C15, Figure 6). This apparently strict identity criterion was chosen because, when using lower identity values such as 99% it was not possible to assign most of the clones to just one cyanobiont type, that is, many of the clones were 99% identical to more than one lichenized cyanobiont.

The number of available non-lichenized cyanobionts detected from the substrates represented, in both matrix types, only around 14% of the total clones. The reason of this low coverage might be due to the primers used (Nübel *et al.*, 1997), which were originally designed to amplify 16S rRNA genes from cyanobacteria in general, not just from *Nostoc* strains. To our knowledge, there are currently no specific primers designed to specifically amplify the 16S rRNA gene of *Nostoc* strains from environmental samples but, given that it was possible to compare the sequences obtained from the clone libraries with those previously obtained from the lichen samples, the aforementioned ones were nonetheless used.

As shown in section 3.3. not all the lichenized cyanobionts were detected in the substrates, with those that were not detected corresponding to the ones whose

abundances were very low in the lichens. Therefore, it is possible that these cyanobionts are present in the substrates but given their low concentrations are unlikely to be detected. Whatever the case, our methodology permitted the comparison of the available non-lichenized potential cyanobionts in the substrates between the different sampling-sites, and the design of more specific primers was not necessary.

In most cases abundance was higher in the lichens than in the substrates, as was expected given that the DNA extracted from the lichens is enriched in cyanobionts, while in the DNA from the substrates there are several other cyanobacterial genomes competing for amplification. However, their apparent rarity in the substrate can also mean that they have a greater fitness in symbiosis than when free-living (Law & Lewis, 1983).

It is interesting to notice that the only non-lichenized potential cyanobionts detected in the substrates were those that were also lichenized in that site, i.e., the cyanobionts that were not present in the lichens from a determined sub-site were also not detected in the respective substrates. One explanation is that the substrates might be enriched in the cyanobionts from the lichens growing in that site, because lichen thalli are a recognized source of photobionts in horizontal transmission or in photobiont switching (Honegger 1993; Paulsrud, 2001; Honegger, 2008). An alternative explanation is that, given their presence in the substrate, these specific cyanobionts were available for lichenization, which would explain their high abundance in symbiosis. Whatever the case, neither can be demonstrated with the present data and, therefore, both alternatives must be considered.

Discussion

The only cyanobiont OTU present in Deception, C14, was also present in the other three sampling sites (Coyhaique, Karukinka and Navarino), being the only one detected in the four locations. Within these sites it was considerably more abundant in DH and NG, which might be considered the most extreme environments from this study given their high latitudes and un-forested characteristics. Because C14 was closely related to a *Nostoc* cyanobiont from Scotland (Kaasalainen *et al.*, 2012), it seems likely that this is a versatile strain, capable of adapting to different ecological conditions such as the ones present in Scotland (similar to the ones from Southern Chile) versus the ones from Antarctica.

Despite the high richness of *Nostoc* clones in certain sub-sites (Table 5), the richness of the available non-lichenized potential cyanobionts was not always correlated with those values. The most extreme case was observed in DH, where 29 different *Nostoc* clone types were found, but only 1 of them (C14) corresponded to a cyanobiont detected in the lichen samples.

As mentioned earlier, the available cyanobionts were defined as the non-lichenized potential cyanobionts present in the substrates plus the lichenized cyanobionts in each sub-site. As was done in the case of the lichenized symbionts, the diversities of the total available cyanobionts that were calculated for each sub-site (Table 6) were first grouped and averaged according to the forest cover of the sub-sites (forested and un-forested) and then, only the forested matrices, grouped and averaged according to their geographic origin (Coyhaique, Karukinka and Navarino). In the first case, the forested and the un-forested matrices presented similar diversities of their available potential cyanobionts and, in the second case, no linear tendency was observed which could relate diversity

with the latitude gradient. This seems to go against the studies suggesting that the diversity of species is higher at lower latitudes (Willig *et al.*, 2003; Dowle *et al.*, 2013); however, this is a controversial topic, because many reports support this conclusion while others have found no relationships between the diversity of unicellular organisms and latitude (Mourelle & Ezcurra, 1997; Fenchel & Finlay, 2004) or have even found opposite patterns with respect to LBG (Andrew & Hughes, 2005; Qian & Ricklefs, 2007; Giordani *et al.*, 2012).

4.3. Mycobiont specificity

Most previous comparative studies of symbiotic specificity (Smith & Douglas, 1987; Beck *et al.*, 2002; Yahr *et al.*, 2006) have used the range of partners a symbiont can associate with as a measure of specificity, which, in its most basic sense, can be considered as the number of symbiotic partners a symbiont can associate successfully with (Poulin *et al.*, 2011). The main problem is that not all symbiotic partners are equal, and this simple number does not capture the differences that exist among symbiotic pairs or the taxonomic (phylogenetic) distances that one symbiont had to "overcome" to associate with different pairs. For that reason, in the present study the genetic relatedness between the cyanobionts was also included as a factor, taking into account their taxonomic affinities. As in the case of diversity, explicit inclusion of the genetic distance alleviates the potential discrepancy that could be generated by mere counts, the ambiguities generated by narrative descriptions and the errors that could be generated by the operational definition of a taxonomic unit (Ricotta *et al.*, 2002; Guiaşu & Guiaşu, 2003; Leinster & Cobbold, 2012). The proposed formula for its calculation (see [4] and

[5], section 3.5) was designed in this work as an adaptation of the *phylogenetic specificity* concept initially presented by Poulin & Mouillot (2003) for host and parasite interactions, who proposed that host specificity should not just include the number of species successfully used by a parasite, but also consider how closely related they are. The greater the taxonomic distinctness between host species, the higher the value of the index, i.e., a high index value means that on average the hosts of a parasite species are not closely related and, therefore, specificity is lower. Given that this idea is highly generalizable, we considered it should be suitable for determining and comparing the specificity of lichen associations. In this work it was named *Phylogenetic local specificity*, where the term "local" refers to the fact that specificity is a concept that should consider all the partners a mycobiont has been found associated with on a global scale, and this work only considered data from some localities in Southern Chile and Antarctica.

It is known that bipartite *Peltigera* species are highly specialized for their cyanobionts at higher taxonomic scales (all bipartite *Peltigera* species are restricted to a single cyanobacterial genus, *Nostoc*), but the specificity degree for these lichens at lower taxonomic levels has been rarely established (Paulsrud, 2001; O'Brien *et al.*, 2005).

In this work, mycobionts M3 (*Peltigera sp.*) and M7 (*P. neckeri* lineage) only interacted with a single cyanobiont type each (C8 and C14, respectively). This situation was discussed by Poulin & Mouillot (2003), who said that the only apparent weakness of their index was that it cannot be applied to parasite species infecting only 1 host species, since there is no pair of host species in these cases from which an average taxonomic distance could be calculated. They proposed 2 ways of dealing with such highly host-

specific parasites. First, they could be excluded from any comparative analysis in which the index is computed or, second, they could be assigned the maximum specificity value, since by definition "all" host species for such specialized parasites belong to the same taxonomic group. In this study the second alternative was adopted and they were given the maximum specificity value. Nevertheless, due to the small sample sizes presented by these "highly specific" mycobionts (n=1 and n=3, respectively) the strength of these inferences is limited and it is possible that they might associate with more cyanobionts than the ones detected in the samplings. However, it must also be kept in mind that these high specificity values decrease the chance of finding suitable photobionts (Fedrowitz, 2011), explaining their scarce or null reports in South America (Martínez *et al.*, 2003). Furthermore, the cyanobiont associated with M3 (C8) was exclusively associated with this mycobiont ("one to one" association), which also lowers the possibility of finding this specific cyanobiont and re establishing the symbiotic association.

Surprisingly, the mycobiont from Deception Island (M2, related to *P. extenuata*) did not present high specificity, associating with 4 cyanobionts. This is rather interesting because these specimens had an asexual (symbiotic) reproductive strategy (with soredia and lacking apothecia), which should imply a higher specificity level, since both symbionts are passed together from one generation to the other. In fact, results obtained by Otálora *et al.* (2013), demonstrated that the reproductive strategies of *Degelia plumbea* and *D. atlantica* significantly affect the genetic diversity of lichenized cyanobionts, because sexual reproduction promotes horizontal transmission, whereas symbiotic reproduction favors the clonal transmission of the symbionts in lichens. However, photobiont switching is known to occur in vegetatively dispersing lichens (eg.

Nelsen & Gargas, 2008; Nelsen & Gargas, 2009; Wornik & Grube, 2010) and given that selectivity patterns depend on various factors such as mycobiont taxa, the environment they encounter, species reproductive strategies and photobiont availability (Fedrowitz, 2011), M2 probably displays a different selectivity for its cyanobionts, being able to change its symbiotic partner depending on the environment in which they are living.

In brief, results show that mycobionts presented different, moderate to high, specificity levels and several of them shared their cyanobionts when living at the same geographic locality. Concerning this last thing, Rikkinen's model of photobiont-mediated guilds (Rikkinen, 2003; Rikkinen, 2013) described that epiphytic communities of *Nephroma* and *Parmeliella* are hypothesized to rely on a common pool of cyanobacterial symbionts and it could be expected a similar situation to be happening in terricolous cyanolichens like *Peltigera.* However, even though most of the cyanobionts were shared among the mycobionts, some *Peltigera* specimens from this study housed their own specific *Nostoc* strains without sharing them with each other; such was the case of M2, M3, M4 and M8, being M3 and M8 the most extreme example since all their cyanobionts were exclusive and did not associate with any of the other mycobionts (Figure 13). This supports the idea that phylogenetically-determined specificity is the primary explanatory factor in symbiotic communities, but within those boundaries, it is the combination of the fungal and cyanobiont partners which determines local fitness and therefore local abundance, manifested as selectivity (Yahr *et al.*, 2006).

pairs present in each sub-site, the one that presented the highest value was DH, the most extreme environment; while KM, one of the least extreme sub-sites from this study, was the site where the average success of the symbiotic pairs was lowest. Concerning this, some studies conducted in arctic regions have determined that rockiness, which was characteristic in the volcanic hillside of DH, is positively related to lichen abundance (Holt *et al.*, 2007). According to these studies very rocky soils suppress vascular plant cover, favouring the development of lichens and bryophytes, which normally are poor competitors against vascular plants but tolerate high stress environments where low temperatures and low water availability reduce or exclude vascular plant cover (Grime, 1977). Furthermore, the deep moss mat development commonly observed in forests appears to disfavor lichen's abundance (Bonan & Shugart, 1989; Viereck & Schandelmeier, 1980), explaining the apparent lower success of the symbiotic pairs in the forested sub-sites.

However, it must be kept in mind that all these studies have been performed considering the lichen as a whole, where the same mycobiont in different specimens indicates that they correspond to the same species, even if they present different photobionts; while in the present study the ecological success was determined for the holobionts, that is, taking into account both symbionts. In any case, when the average success data of the holobionts per sub-site were grouped and averaged according to their matrix type, no differences were observed between the forested and un-forested ones, despite the individual differences above mentioned.

Similarly, there were no differences between the average ecological successes of the symbiotic pairs according to the latitude of their geographic origin, possibly because the

4.4. Ecological success

In many symbioses there is often a range of potential symbiont combinations. However, even compatible associations in one habitat may not be optimal in another, leaving only a sub-set of the total possible associations detectable at any site (Bubrick *et al.*, 1985). Therefore, the frequency of associations in a site is not necessarily determined by the frequency of those partners that arrive and survive by themselves, but rather by the environmentally determined success of the partnerships that they form, in the case of lichens, the holobionts (Yahr *et al.*, 2006). In this work this concept was termed ecological success and, in practice, corresponded to the abundance of the different symbiotic pairs in the environment in which they are present, being therefore the observable result of the combination between the specificity of a determined mycobiont, the environmental availability of its specific cyanobionts and the selectivity of that mycobiont for some of these specific cyanobionts in that environment.

When the ecological success was determined for each symbiotic pair in the different sub-sites, the effect of the environment was observed in the case of those pairs that were present in both matrix types. For example, M4-C7 was clearly more successful in KG (Karukinka Grassland) than in KM (Karukinka Mature-forest), while M5-C1 presented the opposite behaviour, being more successful in CF1 (Coyhaique Forest 1) than in NG (Navarino Grassland). This is not surprising, because mycobiont M4 was related with *P. rufescens*, a species that is more commonly found in open, often exposed, sites; while M5 was related with *P. canina* lineage, whose species normally occur in somewhat sheltered sites (Goward *et al.*, 1994; Brodo *et al.*, 2001).

Interestingly, when calculating the average ecological success of all the symbiotic

latitudinal gradient of the sampling sites was not enough to observe significant differences between them. However, as shown in section 3.2., there was in fact a linear tendency relating the diversity of the mycobionts and the origin of the samples, indicating that the LBG might have a stronger effect on the richness of the different lichens than on their abundance. In fact, LBG has been generally related to an increase in species *richness* from the poles to the tropics (e.g. Gaston, 2000; Willig & Bloch, 2006; Dowle *et al.*, 2013; Rolland *et al.*, 2014), mainly due to faster speciation and reduced extinction in the tropics (Rosenzweig, 1992, Gaston, 2000, Buzas *et al.* 2002; Rolland *et al.*, 2014).

4.5. Mycobiont selectivity

In this study, strong association patterns with respect to cyanobiont availability were determined (G-test: observed vs. expected; $p<0.0001$), suggesting the existence of active processes beyond those with a stochastic explanation.

With the aim of confirming and quantifying the existence of selectivity from the mycobionts, an "electivity index" previously developed for evaluating the utilization by animals of different types of food (Vanderploeg & Scavia, 1979) was adapted for its use in lichen associations. Among the existing indices, Vanderploeg and Scabia's E^* index was shown to be the single most useful one, reaching most of the desirable characteristics of an electivity index (Lechowicz, 1982). The original index relates the selected food types with respect to the available ones, also considering the number of kinds of food available in the environment. In the present work, the selected food types were replaced by the abundance of the symbiotic pairs formed by each mycobiont, the

available food types by the abundance of the available cyanobionts, and the number of kinds of food by the richness of the specific cyanobionts available. The genetic distance between the specific cyanobionts was also incorporated, as was done in the calculations of diversity and specificity. To our knowledge, there are currently no selectivity indices for quantitatively determining selectivity in lichen associations, which makes our index a valuable tool for comparative purposes.

Given that the index adopts a value of cero for a random selection, and a possible range from plus one (+1) to minus one (-1) depending if the photobiont is considered preferred or avoided (analogous to what was described by Vanderploeg & Scavia, 1979), the average selectivity of each mycobiont was calculated using the absolute values of their individual selectivities. This was done because both preference and avoidance correspond to high selectivity cases.

It is not clear what drives selectivity, but there is a developing body of evidence suggesting that it depends on geographical and ecological factors (Piercey-Normore, 2006; Yahr *et al.*, 2006, Škaloud & Peksa, 2010; Werth & Sork 2010) and for that reason, the average selectivity of the mycobionts in the different sub-sites was also calculated. Despite being the most extreme sub-site from this study, selectivity was not determined for the mycobionts in Deception. The reason was that selectivity corresponds to a "choice" of the mycobiont for some of its specific cyanobionts, and given that in DH only a single cyanobiont type (C14) was available for lichenization, it would not be correct to calculate selectivity since there is not an actual selection. From the other sub-sites, the mycobionts resulted to be least selective in KG, the un-forested matrix from Karukinka and, surprisingly, most selective in NG, an ecologically similar un-forested

matrix corresponding to the grassland of Navarino.

Several reports suggest that in more extreme habitats lichens tend to show lower selectivity towards their photobionts (Romeike *et al.*, 2002; Wirtz *et al.*, 2003; Domaschke *et al.*, 2012; Pérez-Ortega *et al.*, 2012) while in temperate and tropical regions they have shown to be highly selective (Helms *et al.*, 2001; Beck *et al.* 2002; Yahr *et al.*, 2004; Hauck *et al.*, 2007). However, these studies were not specifically examining lichens from regions with an extreme climatic regime (Lindblon & Sochting, 2013). In the case of high selectivity, preferences of the photobiont for environmental factors may limit the ecological niches available to lichens (Peksa & Škaloud, 2011) while low selectivity could be an evolutionary advantage in extreme climate regimes where lichens might be utilizing local, better adapted photobionts, rather than restricting themselves to specific types (Fernández-Mendoza *et al.*, 2011).

Therefore, the sampling sub-sites were classified according to two major categories: matrix cover (with or without forest cover) and latitude. To determine if the selectivity of the mycobionts was influenced by the first or the second, the selectivities of the mycobionts per sub-site were grouped and compared first according to their matrix cover and second, only in the case of the forested matrices, according to the geographic origin of the samples. The average selectivities instead of the individual selectivities per mycobiont were used because not all the mycobionts were present in both types of matrix or in the three forested sampling sites.

The average selectivity of the mycobionts was similar in the forested and in the un-forested matrices (section 3.7.), indicating that there was no relation between selectivity and the environmental context in which the lichens were growing. A possible

explanation might be that, given the proximity of the sites with forested and with un-forested matrices, there could be a common pool of cyanobionts adapted to both matrix types from which terricolous lichens such as *Peltigera* could choose, despite the different forest cover. This is different to what was previously observed for epiphytic lichens, which presented high selectivities in forests (e.g. Myllys *et al.*, 2007), maybe because trees might be a more discontinuous environment than soil. However, even though, to our best knowledge there are no studies relating selectivity with forested and un-forested matrices, it has been observed that environments with more marked environmental gradients, such as altitude changes, do actually relate with higher selectivity values (Vargas Castillo & Beck, 2012).

When selectivity was evaluated per sampling site (different latitudes) with similar environmental context (only forested matrices), there was no relation between selectivity and the latitudinal gradient of the sites. This might be explained because all the sites considered in this comparison, despite being in different latitudes, overall correspond to high latitude localities (Southern Chile). So, it cannot be ruled out that expanding the latitude gradient and comparing them with lower latitude sites, differences in selectivity might be observed. In fact one of the advantages of the index presented in this study is that it should permit such quantitative comparisons. Even though Deception was not included in the comparisons, it is interesting to notice that the combination M2-C14 only existed in DH, despite the availability of both partners elsewhere. This could indicate some sort of adaptation of both symbionts to live together as a holobiont in the adverse Antarctic conditions, which might not be the best symbiotic combination in other ecological contexts (Cowan *et al.*, 2014).

Discussion

Molecular biological studies have shown that the selectivity of numerous lichen mycobionts towards their respective photobionts exhibits a continuum of intensity, both on specific and generic levels (Helms *et al.*, 2001; Piercey-Normore & de Priest, 2001; Beck *et al.*, 2002; Romeike *et al.*, 2002). For instance, mycobionts of the genus *Physcia* have been shown to have high selectivity towards their photobionts (Helms *et al.*, 2001), while lower selectivity has been observed in the mycobiont of two species of *Umbilicaria* (Romeike *et al.*, 2002). Ahmadjian *et al.* (1980) further suggested that the mutual compatibility of two potential symbionts for the chloro-lichen *Cladonia cristatella* is defined not only at the generic level, but that the mycobiont of this lichen also requires characters selective at the species level in its choice of photobiont. The variable fitness of symbiont associations across a range of ecological settings may determine their success, and therefore their frequency of association or ecological success in a site or habitat (Fox & Morrow, 1981). However, in most studies selectivity has not been quantitatively measured and it is still not completely distinguished from specificity (Beck *et al.*, 2002; Yahr *et al.*, 2006; Vargas Castillo & Beck, 2012; Jones *et al.*, 2013), making comparative studies even more complicated. Also, to our best knowledge, there are still no reports that assess selectivity considering the available potential photobionts determined directly from the substrates where the lichens grow, which might be the only way to effectively determine if what is considered high selectivity is in fact that, or just a lack of availability of certain potential cyanobionts at a determined environment.

Finally, when calculating selectivity per mycobiont, an inverse relation between selectivity and the ecological success of the mycobionts was observed (Table 10),

suggesting that high selectivities might decrease the chance of finding suitable photobionts and therefore, establish successful lichen associations (Fedrowitz, 2011). On a more global scale, the least selective mycobionts corresponded to the OTU associated with *P. hymenina* lineage. This group includes some rather abundant and widely distributed species, which are present in at least three different continents, mainly along circumpolar or circumboreal Holarctic areas (Martínez *et al.*, 2003). The next least selective mycobiont was the OTU associated with *P. rufescens,* which also presents a world-wide distribution, and has been defined as a cosmopolitan species (Martínez *et al.*, 2003). On the contrary, the most selective mycobionts OTUs were associated with *P. frigida*, a very rare species restricted to Tristan da Cunha (British overseas territory) and Tierra del Fuego (Martínez *et al.*, 2003), and with *P. ponojensis*, which has only been reported in Holarctic regions with no previous reports in South America (Martínez *et al.*, 2003; Quilhot *et al.*, 2012). These results agree with previous studies suggesting that levels of selectivity correlate with the lichen geographic distribution, since low selectivity apparently allows widespread lichen-forming fungi to establish successful symbioses with locally adapted photobionts in a broader range of habitats (Muggia *et al.*, 2014).

CONCLUSIONS

1. Some mycobionts were related with species that have scarce or null reports in South America, which makes the present work a valuable contribution to the knowledge of lichen diversity in Southern Chile and Antarctica.
2. Cyanobiont C14 was the most common and the only one detected in the 4 sampling sites, suggesting that it might correspond to a versatile strain capable of adapting to different ecological conditions.
3. Mycobionts were more diverse in the forested matrices than in the un-forested ones, confirming that forest degradation negatively affects lichen diversity. On its part, cyanobiont diversity was similar in both matrix types, suggesting that other factors such as specificity and selectivity of the mycobionts might be more important than cover type in its determination.
4. On average, the mycobionts were less diverse at higher latitudes, agreeing with the concept of *latitudinal biodiversity gradient* (LBG). The absence of an LBG in the case of the cyanobionts agrees with the idea that this might be weak or even absent in unicellular organisms.
5. None of the symbionts of the pair M3C8 was robustly associated with previously described *Peltigera* and *Nostoc* sequences. This, along with its low ecological success, suggests that it corresponds to a rare un-described species, whose low abundance and lack of previous reports might be explained by its high specificity ("one to one" association).

6. The differences observed in some of the symbiont association patterns with respect to the availability of the cyanobionts suggest the existence of active selection processes. For that reason, the adaptation of an index for quantitatively determining the selectivity of lichen associations constitutes a novelty tool for comparative purposes.
7. The least selective mycobionts were the most successful ones and were related with cosmopolitan species, while the most selective ones were the least successful and were related with species that have narrower global distributions. This agrees with recent reports suggesting that high selectivities might restrict the ability of lichens for colonizing different environments.
8. Contrary to what was proposed in the hypothesis, no relation was observed between the average selectivity of the mycobionts and the type of matrix where they were growing. Likewise, mycobiont selectivity was not related with the latitude of the sampling sites.

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SUPPLEMENTAL MATERIAL

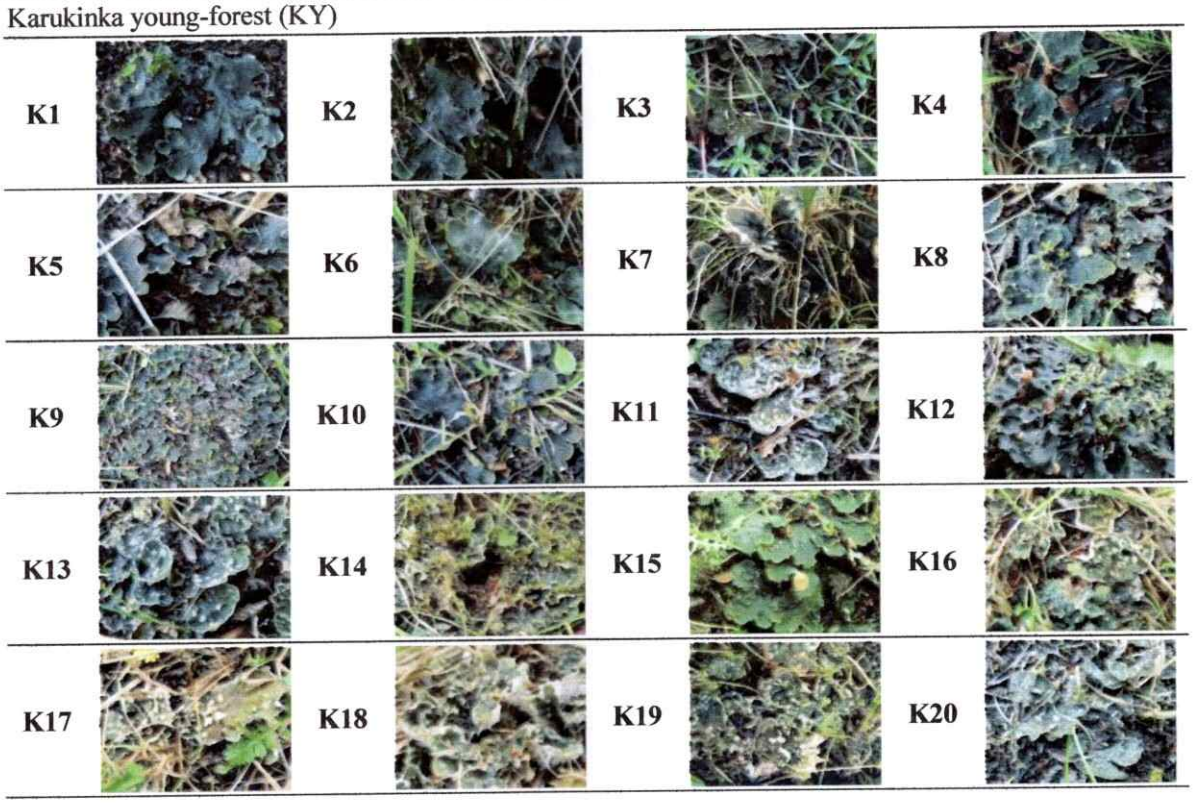
Table S1. Photographs of the samples in the different sub-sites

Coyhaique Forest 1 (CF1)							
C1		**C2**		**C3**		**C4**	
C5		**C6**		**C8**		**C9**	
C10		**C11**		**C12**		**C13**	
C14		**C15**		**C16**		**C17**	
C18		**C19**		**C20**		**C21**	
C22		**C23**		**C24**		**C25**	
C26		**C27**					

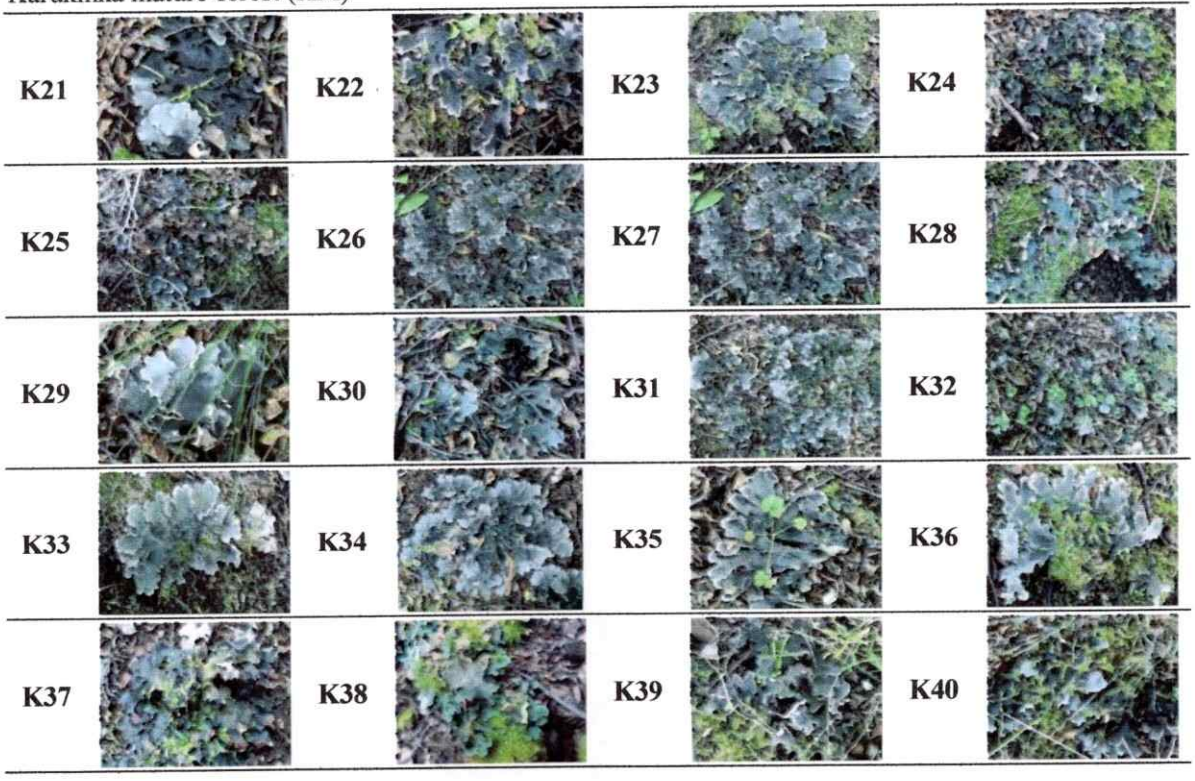
Coyhaique Forest 2 (CF2)

C28		**C29**		**C30**		**C31**	
C32		**C33**		**C34**		**C35**	
C36		**C37**		**C38**		**C39**	
C40		**C41**		**C42**		**C43**	
C44		**C45**		**C46**		**C47**	
C48		**C49**		**C50**		**C51**	
C52							

Karukinka young-forest (KY)



Karukinka mature-forest (KM)



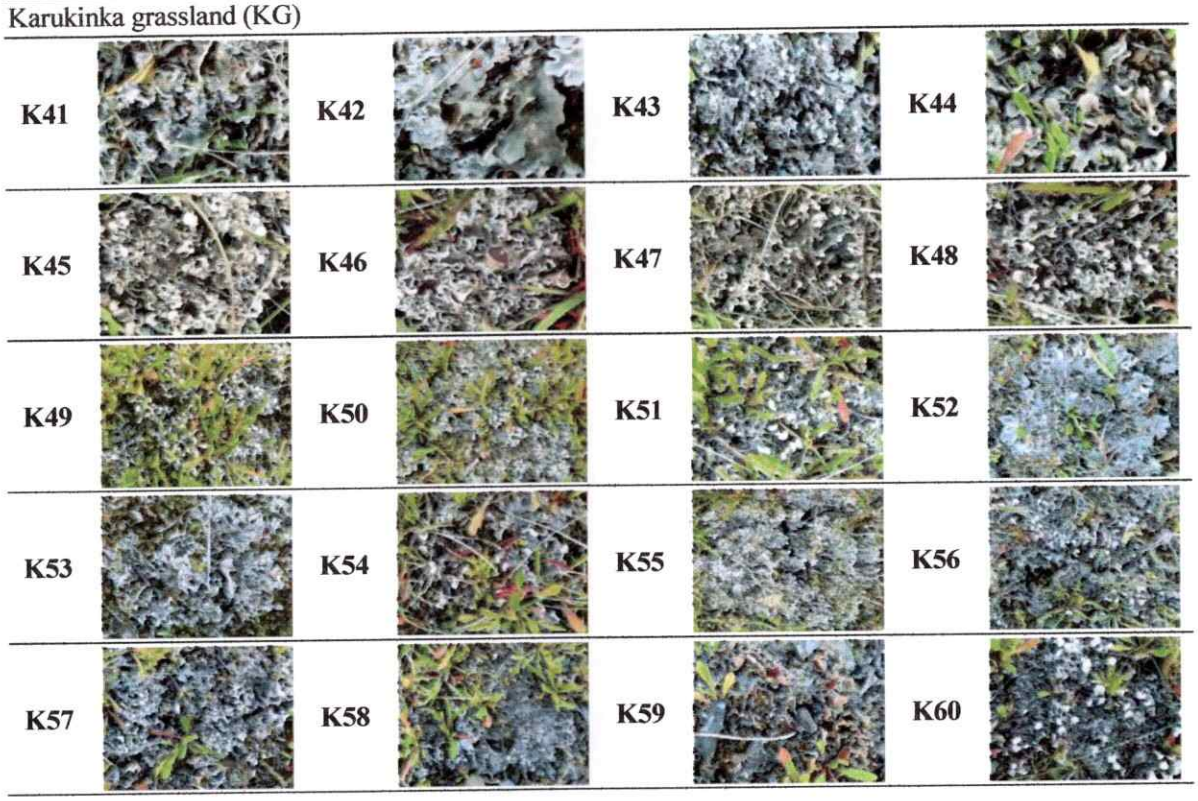
Navarino young-forest (NY)

N1		**N2**		**N3**		**N4**	
N5		**N6**		**N7**		**N8**	
N9		**N10**		**N11**		**N12**	
N13		**N14**		**N15**		**N16**	
N17		**N18**		**N19**		**N20**	

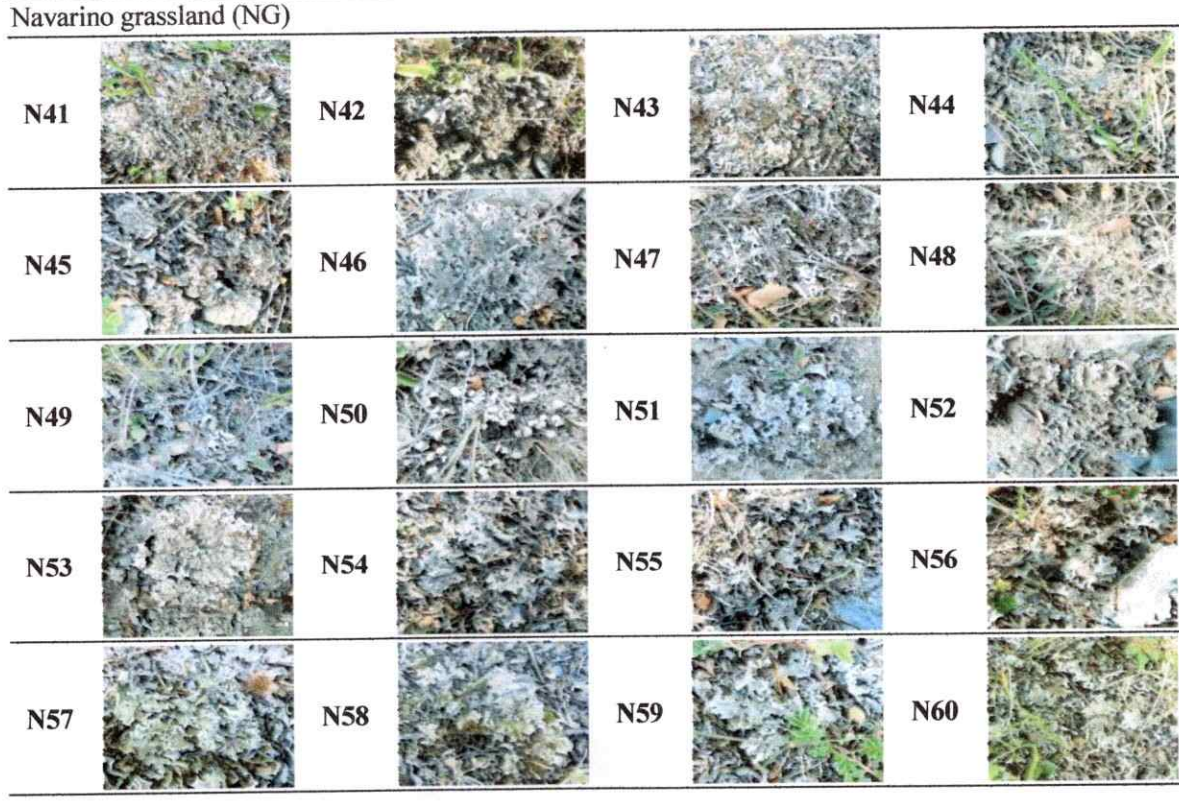
Navarino mature-forest (NM)

N21		**N22**		**N23**		**N24**	
N25		**N26**		**N27**		**N28**	
N29		**N30**		**N31**		**N32**	
N33		**N34**		**N35**		**N36**	
N37		**N38**		**N39**		**N40**	

Karukinka grassland (KG)



Navarino grassland (NG)



Deception hillside (DH)

D1		**D2**		**D3**		**D4**	
D5		**D6**		**D7**		**D8**	
D9		**D10**		**D11**		**D12**	
D13		**D14**		**D15**			

Table S2. Sampling sites, samples and sequence information.

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	**Environmental Context**	**Sample**	**Latitude (S)**	**Longitude (W)**	**Altitude**	**LSU rDNA**	**SSU rDNA**
Coyhaique (C)	*Nothofagus pumilio* young-forest	C1	-45,527495	-72,033067	654,8 m	KF718515	KF718389
	Nothofagus pumilio young-forest	C2	-45,527590	-72,032775	688,6 m	KF718516	KF718390
	Nothofagus pumilio young-forest	C3	-45,527590	-72,032817	688,5 m	KF718517	KF718391
	Nothofagus pumilio young-forest	C4	-45,527640	-72,032805	696,4 m	KF718518	KF718392
	Nothofagus pumilio young-forest	C5	-45,527840	-72,032802	717,6 m	KF718519	KF718393
	Nothofagus pumilio young-forest	C6	-45,527687	-72,033257	709,8 m	KF718520	KF718394
	Nothofagus pumilio young-forest	C8	-45,527767	-72,033582	725,6 m	KF718521	KF718395
	Nothofagus pumilio young-forest	C9	-45,527697	-72,033745	712,4 m	KF718522	KF718396
	Nothofagus pumilio young-forest	C10	-45,527677	-72,033752	708,5 m	KF718523	KF718397
	Nothofagus pumilio young-forest	C11	-45,527770	-72,033735	713,4 m	KF718524	KF718398
	Nothofagus pumilio young-forest	C12	-45,527662	-72,033667	679,0 m	KF718525	KF718399
	Nothofagus pumilio young-forest	C13	-45,527695	-72,034010	717,4 m	KF718526	KF718400
	Nothofagus pumilio young-forest	C14	-45,527637	-72,034177	712,7 m	KF718527	KF718401
	Nothofagus pumilio young-forest	C15	-45,527640	-72,034165	710,8 m	KF718528	KF718402
	Nothofagus pumilio young-forest	C16	-45,527640	-72,034220	711,3 m	KF718529	KF718403
	Nothofagus pumilio young-forest	C17	-45,527675	-72,034190	713,0 m	KF718530	KF718404
	Nothofagus pumilio young-forest	C18	-45,527462	-72,034410	724,1 m	KF718531	KF718405
	Nothofagus pumilio young-forest	C19	-45,527435	-72,034137	724,2 m	KF718532	KF718406
	Nothofagus pumilio young-forest	C20	-45,527682	-72,034802	756,6 m	KF718533	KF718407
	Nothofagus pumilio young-forest	C21	-45,527642	-72,034835	729,8 m	KF718534	KF718408
	Nothofagus pumilio young-forest	C22	-45,527555	-72,035065	694,3 m	KF718535	KF718409
	Nothofagus pumilio young-forest	C23	-45,527477	-72,035210	697,2 m	KF718536	KF718410
	Nothofagus pumilio young-forest	C24	-45,527652	-72,035597	727,1 m	KF718537	KF718411
	Nothofagus pumilio young-forest	C25	-45,527497	-72,035997	709,8 m	KF718538	KF718412
	Nothofagus pumilio young-forest	C26	-45,527588	-72,036150	695,9 m	KF718539	KF718413

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	**Environmental Context**	**Sample**	**Latitude (S)**	**Longitude (W)**	**Altitude**	**LSU rDNA**	**SSU rDNA**
	Nothofagus pumilio young-forest	C27	-45,527346	-72,036335	720,7 m	KF718540	KF718414
	Nothofagus pumilio young-forest	C28	-45,530000	-72,026762	705,4 m	KF718541	KF718415
	Nothofagus pumilio young-forest	C29	-45,529782	-72,026792	713,9 m	KF718542	KF718416
	Nothofagus pumilio young-forest	C30	-45,529692	-72,026937	708,9 m	KF718543	KF718417
	Nothofagus pumilio young-forest	C31	-45,529690	-72,026915	708,9 m	KF718544	KF718418
	Nothofagus pumilio young-forest	C32	-45,529627	-72,027347	704,6 m	KF718545	KF718419
	Nothofagus pumilio young-forest	C33	-45,529802	-72,027360	704,5 m	KF718546	KF718420
	Nothofagus pumilio young-forest	C34	-45,529910	-72,027467	705,1 m	KF718547	KF718421
	Nothofagus pumilio young-forest	C35	-45,529882	-72,027362	706,6 m	KF718548	KF718422
	Nothofagus pumilio young-forest	C36	-45,529832	-72,027367	704,4 m	KF718549	KF718423
	Nothofagus pumilio young-forest	C37	-45,529760	-72,027592	691,0 m	KF718550	KF718424
	Nothofagus pumilio young-forest	C38	-45,529610	-72,027377	708,5 m	KF718551	KF718425
	Nothofagus pumilio young-forest	C39	-45,529490	-72,027647	707,0 m	KF718552	KF718426
	Nothofagus pumilio young-forest	C40	-45,529450	-72,027647	710,8 m	KF718553	KF718427
	Nothofagus pumilio young-forest	C41	-45,529520	-72,027602	710,4 m	KF718554	KF718428
	Nothofagus pumilio young-forest	C42	-45,529527	-72,027627	703,8 m	KF718555	KF718429
	Nothofagus pumilio young-forest	C43	-45,529455	-72,027792	702,1 m	KF718556	KF718430
	Nothofagus pumilio young-forest	C44	-45,529595	-72,027832	702,8 m	KF718557	KF718431
	Nothofagus pumilio young-forest	C45	-45,529515	-72,027832	710,2 m	KF718558	KF718432
	Nothofagus pumilio young-forest	C46	-45,529797	-72,028022	712,0 m	KF718559	KF718433
	Nothofagus pumilio young-forest	C47	-45,529702	-72,028032	705,0 m	KF718560	KF718434
	Nothofagus pumilio young-forest	C48	-45,529637	-72,028160	702,3 m	KF718561	KF718435
	Nothofagus pumilio young-forest	C49	-45,529600	-72,028220	702,7 m	KF718562	KF718436
	Nothofagus pumilio young-forest	C50	-45,529447	-72,028465	703,0 m	KF718563	KF718437
	Nothofagus pumilio young-forest	C51	-45,529307	-72,029745	715,3 m	KF718564	KF718438
	Nothofagus pumilio young-forest	C52	-45,529927	-72,030522	686,1 m	KF718565	KF718439
Karukinka (K)	*Nothofagus pumilio* young-forest	K1	-54,139677	-68,710203	186,6 m	KC514744	KC514624

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	Environmental Context	Sample	Latitude (S)	Longitude (W)	Altitude	LSU rDNA	SSU rDNA
	Nothofagus pumilio young-forest	K2	-54,139630	-68,709712	171,6 m	KC514745	KC514625
	Nothofagus pumilio young-forest	K3	-54,139668	-68,709812	168,7 m	KC514746	KC514626
	Nothofagus pumilio young-forest	K4	-54,139758	-68,709845	197,2 m	KC514747	KC514627
	Nothofagus pumilio young-forest	K5	-54,139775	-68,710757	182,4 m	KC514748	KC514628
	Nothofagus pumilio young-forest	K6	-54,139718	-68,709743	185,1 m	KC514749	KC514629
	Nothofagus pumilio young-forest	K7	-54,139782	-68,709787	178,3 m	KC514750	KC514630
	Nothofagus pumilio young-forest	K8	-54,139773	-68,710823	197,5 m	KC514751	KC514631
	Nothofagus pumilio young-forest	K9	-54,139792	-68,710827	196,2 m	KC514752	KC514632
	Nothofagus pumilio young-forest	K10	-54,139717	-68,710762	190,8 m	KC514753	KC514633
	Nothofagus pumilio young-forest	K11	-54,139712	-68,710878	194,9 m	KC514754	KC514634
	Nothofagus pumilio young-forest	K12	-54,139690	-68,709768	187,5 m	KC514755	KC514635
	Nothofagus pumilio young-forest	K13	-54,139718	-68,709785	191,4 m	KC514756	KC514636
	Nothofagus pumilio young-forest	K14	-54,139678	-68,709775	189,2 m	KC514757	KC514637
	Nothofagus pumilio young-forest	K15	-54,139730	-68,709760	187,0 m	KC514758	KC514638
	Nothofagus pumilio young-forest	K16	-54,139638	-68,709830	181,5 m	KC514759	KC514639
	Nothofagus pumilio young-forest	K17	-54,139633	-68,709872	183,0 m	KC514760	KC514640
	Nothofagus pumilio young-forest	K18	-54,139642	-68,709863	183,0 m	KC514761	KC514641
	Nothofagus pumilio young-forest	K19	-54,139657	-68,709793	186,6 m	KC514762	KC514642
	Nothofagus pumilio young-forest	K20	-54,139705	-68,709818	190,4 m	KC514763	KC514643
	Nothofagus pumilio mature-forest	K21	-54,126923	-68,709487	169,3 m	KC514764	KC514644
	Nothofagus pumilio mature-forest	K22	-54,126932	-68,709418	171,3 m	KC514765	KC514645
	Nothofagus pumilio mature-forest	K23	-54,126787	-68,709200	170,8 m	KC514766	KC514646
	Nothofagus pumilio mature-forest	K24	-54,126657	-68,709040	168,8 m	KC514767	KC514647
	Nothofagus pumilio mature-forest	K25	-54,126602	-68,709053	162,9 m	KC514768	KC514648
	Nothofagus pumilio mature-forest	K26	-54,126725	-68,709285	161,9 m	KC514769	KC514649
	Nothofagus pumilio mature-forest	K27	-54,126743	-68,709635	169,2 m	KC514770	KC514650
	Nothofagus pumilio mature-forest	K28	-54,126820	-68,709563	170,1 m	KC514771	KC514651

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	Environmental Context	Sample	Latitude (S)	Longitude (W)	Altitude	LSU rDNA	SSU rDNA
	Nothofagus pumilio mature-forest	K29	-54,127087	-68,709597	169,0 m	KC514772	KC514652
	Nothofagus pumilio mature-forest	K30	-54,127133	-68,709558	168,8 m	KC514773	KC514653
	Nothofagus pumilio mature-forest	K31	-54,127107	-68,709558	169,2 m	KC514774	KC514654
	Nothofagus pumilio mature-forest	K32	-54,127305	-68,709583	171,3 m	KC514775	KC514655
	Nothofagus pumilio mature-forest	K33	-54,127305	-68,709583	171,3 m	KC514776	KC514656
	Nothofagus pumilio mature-forest	K34	-54,127153	-68,709772	161,4 m	KC514777	KC514657
	Nothofagus pumilio mature-forest	K35	-54,127148	-68,709327	159,6 m	KC514778	KC514658
	Nothofagus pumilio mature-forest	K36	-54,127113	-68,709312	159,6 m	KC514779	KC514659
	Nothofagus pumilio mature-forest	K37	-54,127083	-68,709270	180,6 m	KC514780	KC514660
	Nothofagus pumilio mature-forest	K38	-54,126927	-68,709337	153,0 m	KC514781	KC514661
	Nothofagus pumilio mature-forest	K39	-54,127305	-68,709613	198,8 m	KC514782	KC514662
	Nothofagus pumilio mature-forest	K40	-54,127270	-68,709775	178,1 m	KC514783	KC514663
	Grassland	K41	-54,126535	-68,709005	146,0 m	KC514784	KC514664
	Grassland	K42	-54,126588	-68,708953	147,2 m	KC514785	KC514665
	Grassland	K43	-54,126577	-68,708882	145,0 m	KC514786	KC514666
	Grassland	K44	-54,126430	-68,708863	152,1 m	KC514787	KC514667
	Grassland	K45	-54,126428	-68,708597	154,0 m	KC514788	KC514668
	Grassland	K46	-54,126385	-68,708490	160,0 m	KC514789	KC514669
	Grassland	K47	-54,126358	-68,708532	159,0 m	KC514790	KC514670
	Grassland	K48	-54,126363	-68,708618	158,6 m	KC514791	KC514671
	Grassland	K49	-54,126367	-68,708592	157,8 m	KC514792	KC514672
	Grassland	K50	-54,126322	-68,708813	154,1 m	KC514793	KC514673
	Grassland	K51	-54,126292	-68,708772	155,9 m	KC514794	KC514674
	Grassland	K52	-54,126317	-68,708877	152,4 m	KC514795	KC514675
	Grassland	K53	-54,126352	-68,708983	151,0 m	KC514796	KC514676
	Grassland	K54	-54,126227	-68,708912	152,4 m	KC514797	KC514677
	Grassland	K55	-54,126258	-68,708945	152,3 m	KC514798	KC514678

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	Environmental Context	Sample	Latitude (S)	Longitude (W)	Altitude	LSU rDNA	SSU rDNA
	Grassland	K56	-54,126237	-68,708807	153,6 m	KC514799	KC514679
	Grassland	K57	-54,126193	-68,708778	156,5 m	KC514800	KC514680
	Grassland	K58	-54,126187	-68,708762	157,3 m	KC514801	KC514681
	Grassland	K59	-54,126265	-68,708748	152,1 m	KC514802	KC514682
	Grassland	K60	-54,126247	-68,708725	149,9 m	KC514803	KC514683
Navarino (N)	*Nothofagus pumilio* young-forest	N1	-54,939233	-67,602461	39,4 m	KF718581	KF718455
	Nothofagus pumilio young-forest	N2	-54,939147	-67,602489	34,8 m	KF718582	KF718456
	Nothofagus pumilio young-forest	N3	-54,939033	-67,602642	33,1 m	KF718583	KF718457
	Nothofagus pumilio young-forest	N4	-54,938972	-67,603481	26,4 m	KF718584	KF718458
	Nothofagus pumilio young-forest	N5	-54,939644	-67,602911	nd	KF718585	KF718459
	Nothofagus pumilio young-forest	N6	-54,939061	-67,603669	48,1 m	KF718586	KF718460
	Nothofagus pumilio young-forest	N7	-54,939069	-67,603250	14,0 m	KF718587	KF718461
	Nothofagus pumilio young-forest	N8	-54,939278	-67,604058	87,8 m	KF718588	KF718462
	Nothofagus pumilio young-forest	N9	-54,939394	-67,603911	44,8 m	KF718589	KF718463
	Nothofagus pumilio young-forest	N10	-54,939194	-67,604011	2,8 m	KF718590	KF718464
	Nothofagus pumilio young-forest	N11	-54,939122	-67,602331	49,3 m	KF718591	KF718465
	Nothofagus pumilio young-forest	N12	-54,939181	-67,601972	63,9 m	KF718592	KF718466
	Nothofagus pumilio young-forest	N13	-54,939033	-67,602719	2,6 m	KF718593	KF718467
	Nothofagus pumilio young-forest	N14	-54,938894	-67,602228	27,7 m	KF718594	KF718468
	Nothofagus pumilio young-forest	N15	-54,938847	-67,603111	nd	KF718595	KF718469
	Nothofagus pumilio young-forest	N16	-54,938844	-67,601986	72,8 m	KF718596	KF718470
	Nothofagus pumilio young-forest	N17	-54,938781	-67,602211	64,3 m	KF718597	KF718471
	Nothofagus pumilio young-forest	N18	-54,938853	-67,602525	20,2 m	KF718598	KF718472
	Nothofagus pumilio young-forest	N19	-54,938806	-67,602172	34,5 m	KF718599	KF718473
	Nothofagus pumilio young-forest	N20	-54,938969	-67,602319	24,6 m	KF718600	KF718474
	Nothofagus pumilio mature-forest	N21	-54,948239	-67,656414	75,2 m	KF718601	KF718475
	Nothofagus pumilio mature-forest	N22	-54,948325	-67,656456	103,3 m	KF718602	KF718476

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	Environmental Context	Sample	Latitude (S)	Longitude (W)	Altitude	LSU rDNA	SSU rDNA
	Nothofagus pumilio mature-forest	N23	-54,948603	-67,655883	95,7 m	KF718603	KF718477
	Nothofagus pumilio mature-forest	N24	-54,948211	-67,654447	119,6 m	KF718604	KF718478
	Nothofagus pumilio mature-forest	N25	-54,948289	-67,655128	121,1 m	KF718605	KF718479
	Nothofagus pumilio mature-forest	N26	-54,948264	-67,654931	86,5 m	KF718606	KF718480
	Nothofagus pumilio mature-forest	N27	-54,948322	-67,654219	122,1 m	KF718607	KF718481
	Nothofagus pumilio mature-forest	N28	-54,948328	-67,654889	116,5 m	KF718608	KF718482
	Nothofagus pumilio mature-forest	N29	-54,948464	-67,654628	139,0 m	KF718609	KF718483
	Nothofagus pumilio mature-forest	N30	-54,948081	-67,653619	33,8 m	KF718610	KF718484
	Nothofagus pumilio mature-forest	N31	-54,948425	-67,655694	9,2 m	KF718611	KF718485
	Nothofagus pumilio mature-forest	N32	-54,948381	-67,653761	164,8 m	KF718612	KF718486
	Nothofagus pumilio mature-forest	N33	-54,948414	-67,654653	113,2 m	KF718613	KF718487
	Nothofagus pumilio mature-forest	N34	-54,948706	-67,653544	74,1 m	KF718614	KF718488
	Nothofagus pumilio mature-forest	N35	-54,948297	-67,654128	135,2 m	KF718615	KF718489
	Nothofagus pumilio mature-forest	N36	-54,948611	-67,652719	51,2 m	KF718616	KF718490
	Nothofagus pumilio mature-forest	N37	-54,948636	-67,647950	31,3 m	KF718617	KF718491
	Nothofagus pumilio mature-forest	N38	-54,948422	-67,648056	45,8 m	KF718618	KF718492
	Nothofagus pumilio mature-forest	N39	-54,948369	-67,647856	49,7 m	KF718619	KF718493
	Nothofagus pumilio mature-forest	N40	-54,948106	-67,648117	79,9 m	KF718620	KF718494
	Grassland	N41	-54,942072	-67,625772	33,1 m	KF718621	KF718495
	Grassland	N42	-54,942019	-67,625511	34,0 m	KF718622	KF718496
	Grassland	N43	-54,941969	-67,625061	29,5 m	KF718623	KF718497
	Grassland	N44	-54,940117	-67,621767	21,4 m	KF718624	KF718498
	Grassland	N45	-54,939853	-67,621586	20,5 m	KF718625	KF718499
	Grassland	N46	-54,939633	-67,621469	65,5 m	KF718626	KF718500
	Grassland	N47	-54,941869	-67,660394	8,8 m	KF718627	KF718501
	Grassland	N48	-54,941814	-67,661047	23,6 m	KF718628	KF718502
	Grassland	N49	-54,941811	-67,660989	24,5 m	KF718629	KF718503

Sampling site information		Sample information (geo-reference data)				Sequence information (accession numbers)	
Site	Environmental Context	Sample	Latitude (S)	Longitude (W)	Altitude	LSU rDNA	SSU rDNA
	Grassland	N50	-54,941894	-67,661139	28,0 m	KF718630	KF718504
	Grassland	N51	-54,941672	-67,661214	35,7 m	KF718631	KF718505
	Grassland	N52	-54,941883	-67,661442	17,9 m	KF718632	KF718506
	Grassland	N53	-54,941839	-67,660272	22,2 m	KF718633	KF718507
	Grassland	N54	-54,939136	-67,601675	46,1 m	KF718634	KF718508
	Grassland	N55	-54,939006	-67,601806	47,4 m	KF718635	KF718509
	Grassland	N56	-54,939175	-67,601947	49,4 m	KF718636	KF718510
	Grassland	N57	-54,939308	-67,602158	45,6 m	KF718637	KF718511
	Grassland	N58	-54,939269	-67,602325	39,0 m	KF718638	KF718512
	Grassland	N59	-54,939289	-67,602439	39,1 m	KF718639	KF718513
	Grassland	N60	-54,939378	-67,602478	42,0 m	KF718640	KF718514
Deception (D)	Volcanic hillside	D1	-62,972691	-60,575246	29,9 m	KF718566	KF718440
	Volcanic hillside	D2	-62,972658	-60,57515	28,1 m	KF718567	KF718441
	Volcanic hillside	D3	-62,97262	-60,575316	23,3 m	KF718568	KF718442
	Volcanic hillside	D4	-62,97264	-60,575283	22,9 m	KF718569	KF718443
	Volcanic hillside	D5	-62,972666	-60,57537	23,6 m	KF718570	KF718444
	Volcanic hillside	D6	-62,972765	-60,575593	24,2 m	KF718571	KF718445
	Volcanic hillside	D7	-62,972758	-60,575615	23,5 m	KF718572	KF718446
	Volcanic hillside	D8	-62,972756	-60,575648	21,6 m	KF718573	KF718447
	Volcanic hillside	D9	-62,972738	-60,575691	22,7 m	KF718574	KF718448
	Volcanic hillside	D10	-62,973043	-60,575723	16,6 m	KF718575	KF718449
	Volcanic hillside	D11	-62,973046	-60,576206	16,2 m	KF718576	KF718450
	Volcanic hillside	D12	-62,973048	-60,576203	16,2 m	KF718577	KF718451
	Volcanic hillside	D13	-62,973055	-60,576195	15,5 m	KF718578	KF718452
	Volcanic hillside	D14	-62,973055	-60,576193	15,5 m	KF718579	KF718453
	Volcanic hillside	D15	-62,973056	-60,576193	15,4 m	KF718580	KF718454