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Genetic diversity of terricolous *Peltigera* cyanolichen communities in different conservation states of native forest from southern Chile

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Summary. Decreasing quality of forest habitats is among the major factors leading to a loss of epiphytic lichen diversity. However, there is little information about how this factor influences the diversity of terricolous lichens, which do not grow over living trees and could be less susceptible to such disturbances. In this work we describe the genetic diversity of *Peltigera* terricolous cyanolichens and their cyanobiont (*Nostoc*) from three habitats at the Karukinka Natural Park (Tierra del Fuego, southern Chile), which represent different conservation states: native mature-forest (low disturbance intensity), native young-forest (medium disturbance intensity) and grassland (high disturbance intensity). In both forest contexts, a higher diversity and a higher number of unique OTUs (operational taxonomic units) were found. In contrast, in the grassland, the diversity was lower and the *Peltigera* species were mostly cosmopolitan. The presence of unique OTUs and the higher diversity of lichens in native forest areas highlight the importance of their preservation, indicating that decreasing forest quality also has a negative impact on terricolous lichens diversity. [**Int Microbiol** 2013; 16(4):243-252]

Keywords: *Peltigera* · *Nostoc* · lichens · genetic diversity · Karukinka Natural Park · southern Chile

Introduction

Cyanolichens, colonize a wide variety of habitats in southern temperate rainforests, mainly because they are capable of performing photosynthesis at low light intensity, rapidly increase their photosynthetic activity when rehydrated, and can fix atmospheric nitrogen, contributing to natural reserves of this element in forests [28]. For these reasons, they have been described as an important component in maintaining the biodiversity of forests, particularly in soils with limited amounts of nitrogen [4,11].

Temperate forests of southern Chile are characterized by high levels of vascular flora endemism [8]. Therefore, habitat degradation, which is caused by natural or anthropogenic disturbances, has become one of the most worrying issues in current ecology due to its impact on biodiversity [39]. The main threats to general biodiversity also apply to lichens, the most important being habitat loss. Additionally, ecological factors

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Fig. 1. Sampling region at Karukinka Natural Park, Tierra del Fuego Island,southern Chile.

such as stand level limit the successful establishment of lichens under certain conditions [26,41], suggesting that lichens would also be affected by habitat degradation and changes in management.

There are several reports supporting the idea that forest fragmentation may influence the diversity of epiphytic lichen communities (i.e. those that grow on plant species) [e.g., 1,3,5,7,13,16,27], since alterations of forests lead to a reduction in the availability of their substrate, isolating the communities and changing microclimatic conditions [17,23]. 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 [25,32,35,38].

Additionally, lichens are considered one of the groups of organisms challenged by the taxonomic impediment [10], for which many parts of the world remain poorly studied. In southern Chile, although certain lichens have been specifically collected [e.g., 6,14,29], many groups are still poorly known and little specialist expertise exists in the country.

The biological model of this study were terricolous members of the genus *Peltigera* growing in different habitats in Tierra del Fuego Island (Chile), in order to determine the relationship between habitat quality and lichen genetic diversity. Most species of *Peltigera* are terricolous foliose cyanolichens (bipartite associations with cyanobacteria of the genus *Nostoc* as photobionts) and are one of the main groups involved in nitrogen and carbon fixation in different ecosystems. These characteristics, together with their ability to survive under stressful conditions at degraded areas, make these cyanolichens good colonizers in nutrient-poor environments and essential for ecological succession processes [31].

Peltigera species are an abundant component of lichen communities in the southernmost forests of the Karukinka Natural Park (Fig. 1), the largest protected area in Tierra del Fuego Island (Chile), which is preserved by the Wildlife Conservation Society (WCS) [33]. This park, which has also the largest area of continuous forests of the Chilean Patagonian region (approx. 150,000 ha), contains most of the diversity of species and habitats in Tierra del Fuego Island, including a diverse cryptogamic flora represented by a great number of mosses and lichens, a high percentage of which are endemic and constitute an important reservoir of diversity [2,8].

Although Karukinka Natural Park is currently a protected area, some primary native forests have suffered anthropogenic disturbances, such as logging or burning, and young forest patches were generated by regrowth of small trees. These secondary young forests are generally a poor habitat for many native species including lichens. In other areas of the park, changes in the forest vegetation are abrupt, generating grasslands, where the habitat loss and the soil degradation are even more severe. This degradation of the native forest produces patches surrounded by degraded areas of grassland [20] with the subsequent generation of landscape heterogeneity and changes in the habitat quality.

The main objective of this study was to determine the genetic diversity of the symbiotic components (mycobiont and cyanobiont) of *Peltigera* lichens in three habitats that represent different disturbance intensities in these southern forests from Tierra del Fuego Island, southern Chile.

Materials and methods

Study site and lichens sampling. The sampling sites were located at "Estancia Vicuña" within the Karukinka Natural Park (approx. 300,000 ha) in the Tierra del Fuego Island, southern Chile (Fig. 1). This park is characterized by remnants of primary forests of "southern beeches" (*Nothofagus pumilio* [Poepp. et Endl.] Krasser, *N. betuloides* [Mirb.] Oerst. and *N. antarctica* [Forster] Oerst.), among other southern ecosystems [33]. An exhaustive sampling was performed on January 2011, when thallus fragments from 150 samples of *Peltigera* lichens separated by at least one meter to each other were collected, more closely to the edge than to the center of each habitat, which represent different conservation states: (i) 50 samples from a mature-forest of *N. pumilio* (low disturbance intensity, M), (ii) 50 from a young-forest of *N. pumilio* (medium disturbance intensity, Y), and (iii) 50 from a grassland with no forest cover (high disturbance intensity, G). On average, the young-forest samples (Y) were at a distance of approx. 500 m from the mature-forest samples (M), which in turn were about 15 m far from the grassland samples (G). Geo-referencing data were taken for each sample point and the samples were stored in paper bags at room temperature until their analysis. After this initial sampling, a new selection from the 150 individuals was performed. The first step consisted in segregating *Peltigera* individuals into phenotypically different groups based on the color of the thallus, presence/absence of reproductive structures, sexual/asexual reproductive structures, rhizines and venation types, even if they were represented by a single individual. Then, the groups were completed with a random selection between the remaining samples, until a final number of 20 individuals per habitat was reached (Y: K1-K20; M: K21-K40; G: K41-K60). This random selection was assessed separately for each habitat and adjusted for the corresponding frequencies of the different morphologically groups. Examples of the different morphotypes from the three habitats are shown in Fig. 2.

Pre-treatment of the samples and DNA extraction. All lichen thalli were superficially cleaned with a sterile brush and a spatula, and then thoroughly rinsed with sterile water. Eighty to 100 mg from each lichen thallus were mechanically fractioned with a mini-grinder, and DNA was extracted with the PowerSoil DNA Isolation kit (MoBio Laboratories, CA, USA) according to the manufacturer's instructions. Quality and integrity of the extracted DNA was 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.

PCR amplifications and sequencing. From the isolated DNA of each lichen thallus, the fungal 18S rRNA gene was amplified with the specific primers EF4 and EF3 [37], whereas the fungal 28S rRNA gene was amplified using the primers LIC24R [21] and LR7 [24]. On the other hand, the cyanobacterial 16S rRNA gene was amplified with the PCR1 and PCR18 primers [42].

All amplifications were performed according to the cited literature recommendations and using the 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) in a Maxygene thermocycler (Axygen, CA, USA). The concentration and quality of the amplicons were determined electrophoretically as described above except that 1.2 % (w/v) agarose gels were used.

The amplicons obtained from each molecular marker were sequenced (Macrogen, Seoul, South Korea) using the forward primers with a Genetic Analyzer 3730XL (Applied Biosystems, CA, USA). The 18S rRNA, 28S rRNA and 16S rRNA genes sequences obtained from this study, 60 per each molecular marker, were deposited in the GenBank database under accession numbers KC514684 to KC514743, KC514744 to KC514803 and KC514624 to KC514683, respectively.

Sequence alignment and phylogenetic analyses. DNA sequences were manually edited with the Mega5 software (available at http://www.megasoftware.net), aligned with the Muscle alignment tool and some ambiguously aligned nucleotides were removed prior to analysis. Sequence fragments obtained from both, the mycobionts and the cyanobionts, were subjected to BLASTN queries for an initial verification of their identities by comparison to the non-redundant nucleotide database at GenBank (NCBI).

Alignments of both sequence sets were subjected to phylogenetic reconstructions using the maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods. MP analyses were performed with the PAUP* 4.0b10 program, using a heuristic search by the tree bisection-reconnection (TBR) algorithm with 10 random additions and retaining 100 trees in each analysis. The statistical confidence of the nodes was evaluated using 1000 bootstrap pseudo-replicates. ML analyses were performed at the PhyML 3.0. online bioinformatic platform (available at http://www.atgc-montpellier.fr/phyml/), selecting the best nucleotide substitution model with the help of the jModelTest 2.1.1 program (available at http://darwin.uvigo.es). The general time-reversible substitution model assuming a gamma distribution (GTR + G) was selected according to the Akaike's information criterion (AIC). BI was carried out using the Metropolis-coupled Bayesian Markov chain Monte Carlo algorithm $(MC)^3$ implemented in the software MrBayes v. 3.1.2 (available at http://mrbayes.sourceforge.net/download.php). Two independent runs of 10 million generations each were made for both symbiont matrices, sampling the chains every 1000 generations.

Fig. 2. Morphotype representatives of the genus *Peltigera*. Violet frames: samples from young forest; red frames: samples from mature forest; green frame: sample from grassland.

Phylogenetic trees were drawn using the program FIGTREE v1.3.1 [available at http://tree.bio.ed.ac.uk/software/figtree/).

For the mycobiont analyses, *Solorina saccata* isolate AFTOL-ID 127 was used as an out-group (accession numbers DQ973021.1 and DQ973044.1 for the 18S rRNA and 28S rRNA genes nucleotide sequences, respectively), while for the cyanobiont analyses the out-group used was the 16S rRNA gene nucleotide sequence of *Anabaena spiroides* strain PMC9403 (accession number AJ293116.1).

Based on the phylogenetic analyses, an operational definition of the different mycobionts and cyanobionts present in the samples was established. The *Peltigera* mycobiont OTUs (PmOs) were defined as the statistically supported monophyletic groups identified from the three phylogenetic analyses performed (maximum-likelihood bootstrap values ≥75 %, Bayesian posterior probabilities ≥95 %, and parsimony bootstrap values ≥75 %); each of which was in addition identified, based on sequence data, as a different *Peltigera* species. On the other hand, the *Nostoc* cyanobiont OTUs (NcOs) were defined as the sequences with a 100% identity or haplotypes, separating the different cyanobionts according just to the nucleotide identity.

Data analyses. In order to ensure that a suitable number of individuals were considered in the analyses, some parameters were calculated based on the mycobiont OTUs detected in each habitat: (i) the coverage estimation $C_x = 1 - (N_x/n)$, where N_x is the number of OTUs and n is the total number of individuals; and (ii) the Margalef index $D_{Mg} = (S-1)/\ln N$, where S is the number of OTUs and N is the total number of individuals collected.

The diversity of the mycobionts and cyanobionts was determined by the weighted Shannon index ($H_w = -\sum w_i\, p_i \log p_i$), where p_i is the proportion between the number of individuals of the same OTU and the total number of individuals; and w_i is a factor that weights the similarity relationship between OTUs, in this case the average of the genetic p-distances between one OTU and the other OTUs present in each of the three habitats.

The relationship between the habitats was estimated based on the weighted frequencies of the mycobionts and cyanobionts using the Bray-Curtis index and the UPGMA algorithm. The correlation between the geographic distances of each individual thallus with respect to the *p*-distances of the components of each cyanolichen, was performed by means of the Mantel test using the PAST tool v2.16.

Results

Sixty fungal 18S rRNA and 28S rRNA genes nucleotide sequences were successfully amplified from the DNA extracted directly from the lichen samples. Both sets were concatenated

and the phylogenetic analyses of MP, ML and BI were performed with a total of 1711 positions in the final dataset. All three methods yielded similar tree topologies with significant and similar support for the groups. Only the best tree obtained from the ML analysis is shown (log-likelihood: –3367.4162; nucleotide frequencies: A = 0.2717, C = 0.1975, G = 0.2756, T = 0.2552) (Fig. 3).

The analysis allowed the establishment of seven monophyletic groups with strong branch support, which were defined as the operational taxonomic units (OTUs) of the mycobionts and named from PmO1 to PmO7. They were identified with a 100% nucleotide identity, using the BLASTN bioinformatic tool, as *P. rufescens* (Weiss) Humb. (PmO1), *P. "fuscopraetex-*

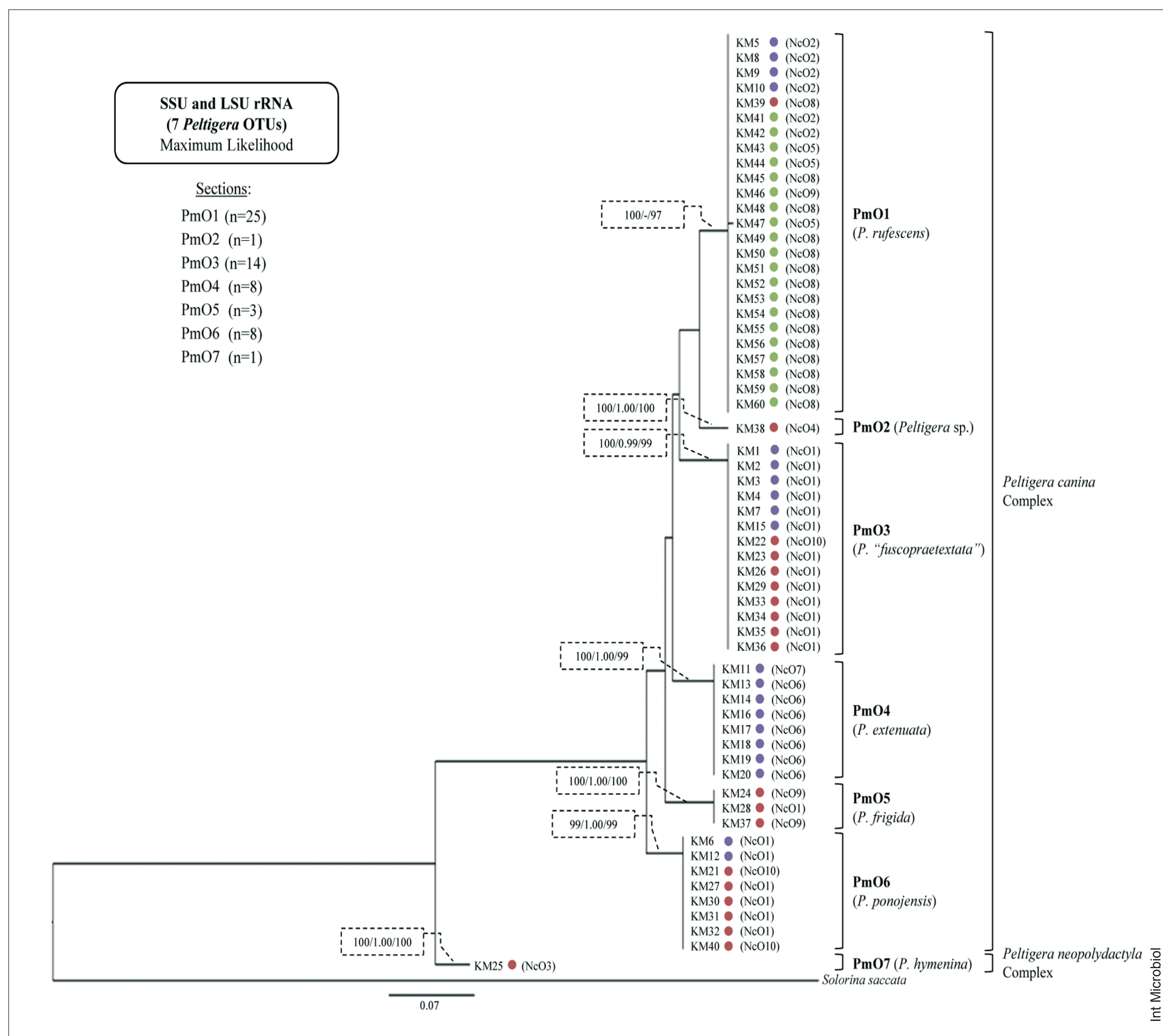

Fig. 3. Phylogenetic relationships among *Peltigera* mycobionts. The phylogeny was based on maximum likelihood analysis of 18S and 28S rRNA genes sequences. Support values are indicated in dashed boxes for nodes that received significant support from at least one method (maximum-likelihood bootstrap values ≥75 %, Bayesian posterior probabilities ≥95%, and maximum-parsimony bootstrap values ≥75 %; ML-BS/pp/MP-BS). KM1-KM20 (violet circles), young forest; KM21-KM40 (red circles), mature forest; KM41-KM60 (green circles), grassland. The names in parentheses next to each sample indicate the *Nostoc* cyanobiont OTU (NcO) associated with each individual mycobiont. The mycobiont OTUs from PmO1 to PmO7 and the species and complex names are indicated next to the brackets.

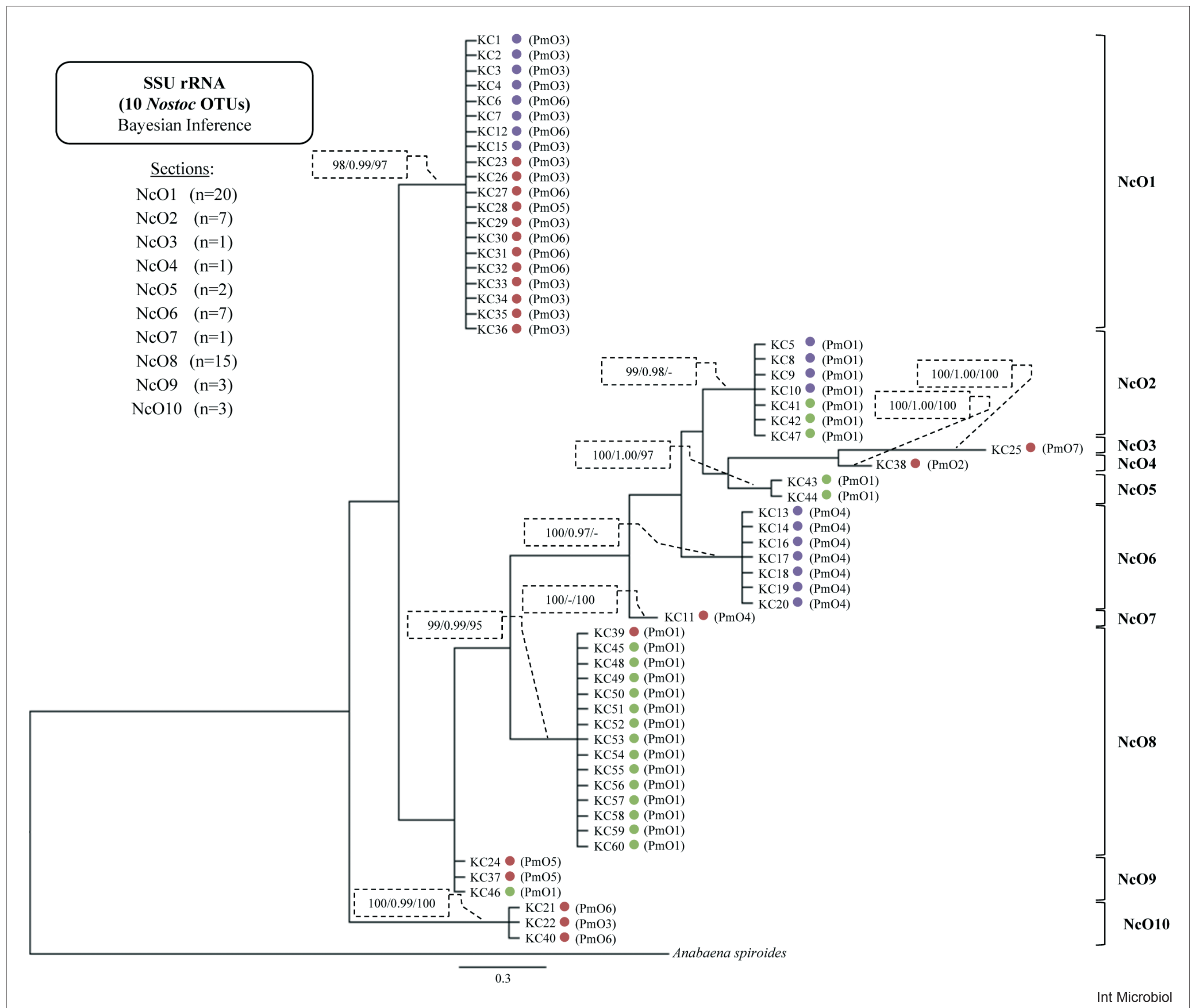

Fig. 4. Phylogenetic relationships among *Nostoc* cyanobionts. The phylogeny was based on Bayesian Inference analyses of 16S rDNA sequences. Support values are indicated in dashed frames for nodes that received significant support from at least one method (maximum-likelihood bootstrap values ≥75 %, Bayesian posterior probabilities ≥95 %, and maximum-parsimony bootstrap values ≥75 %; ML-BS/pp/MP-BS). KC1-KC20 (violet circles): young forest; KC21-KC40 (red circles): mature forest; KC41-KC60 (green circles): grassland. The names in parentheses next to each sample indicate the *Peltigera* mycobiont OTU (PmO) associated with each individual cyanobiont. The cyanobiont OTUs from NcO1 to NcO10 are indicated next to the brackets.

tata" (PmO3), *P. extenuata* (Nyl.) Vain. (PmO4), *P. frigida* R. Sant. (PmO5), *P. ponojensis* Gyeln. (PmO6) and *P. hymenina* (Ach.) Delise ex Duby (PmO7). PmO2, on the other hand, presented some ambiguities on its 18S rRNA and 28S rRNA genes BLAST analyses, so the ITS (Internal Transcribed Spacers including the 5.8S rRNA gene) was used as a third molecular marker (data not shown). The BLAST analysis showed that it might be related to *P. "papuana"* described by Sérusiaux et al. [36]. In addition, its ITS_1-HR [22] was similar to the one presented for the *P. papuanorum* division, but with an insertion of 52 nucleotides.

The analyses showed the existence of two main sister clades, the first one consisted of mycobionts from the *P. canina* complex and included OTUs from PmO1 to PmO6; the other clade consisted on a single OTU, corresponding to PmO7, from the *P. neopolydactyla-dolichorizha* complex [21].

The most abundant OTU was PmO1, with 25 specimens, being the only one present in all three habitats (Y, M and G;

Table 1. Weighted Shannon index of mycobionts and cyanobionts per habitat

	Weighted Shannon Index	
	Mycobionts	Cyanobionts
M	0.0199 ± 0.0006	0.0289 ± 0.0028
Y	0.0115 ± 0.0013	0.0195 ± 0.0019
G	0.0000 ± 0.0000	0.0113 ± 0.0007

M: mature-forest, Y: young-forest, G: grassland. Values are means ± standard deviation.

violet, red and green circles, respectively). The second largest OTU was PmO3, comprising 14 specimens that were present at both forest covered environments (violet and red circles). PmO4 and PmO6 were the third largest groups, with 8 members each; PmO4 was present exclusively at the young forest (violet circles), while PmO6 was present at both forests (violet and red circles). PmO5 was the second smallest OTU with 3 members from the mature forest (red circles); whereas PmO2 was the smallest OTU of this clade, with only one representative from the mature forest (KM38). From the *P. neopolydactyla-dolichorhiza* complex, PmO7 was the only OTU, with a single member from the mature forest (KM25). The weighted Shannon index of mycobiont OTUs was higher in the mature-forest (M), followed by the young forest (Y) and presenting only one mycobiont OTU in the grassland (G) (Table 1).

On the other hand, all 60 cyanobacterial 16S rRNA gene nucleotide sequences were successfully amplified from the lichen samples, and every sequencing reaction produced well-defined reads with no evidence of secondary peaks. A final dataset with a total of 717 positions was subjected to phylogenetic analyses of MP, ML and BI and the three methods yielded similar topologies. Only the tree from the BI analysis is shown since it delivered the best resolution between the groups (Fig. 4).

The analyses allowed the establishment of 10 haplotypes, named from NcO1 to NcO10. All monophyletic groups obtained significant support, except NcO9, whose position was not determined by any of the performed analyses. Despite that, it was still considered as a group, since the nucleotide sequences of the included samples were 100% identical.

There were two main sister clades on the cyanobiont tree. The first included from NcO1 to NcO9, while the second included only NcO10. NcO1 was the largest cyanobiont OTU, with 20 samples present in lichens from both forest-covered environments (violet and red circles). The second largest OTU was NcO8 with 15 samples, 14 of which were found in lichens from the grassland (green circles) and one from the mature forest (red circles). NcO2 and NcO6 were represented by 7 cyanobiont sequences each; NcO2 cyanobionts were from the young forest and the grassland (violet and green circles, respectively), while NcO6 was exclusively from the young forest (violet circles). NcO5, NcO9 and NcO10 comprised 2, 3 and 3 samples, respectively, whereas NcO3, NcO4 and NcO7 were only formed by a single sequence each. In the case of the diversity of cyanobiont OTUs, the weighted Shannon index was higher than that of the mycobionts and the highest diversity was also present in the mature-forest (M), followed by the young-forest (Y) and the grassland (G) showing the lowest diversity (Table 1).

As a measure of the expected species richness, the Margalef index resulted in values of 5.7, 3.7 and 0.7, considering the samples of the mature-forest (M), the young-forest (Y) and the grassland (G), respectively. For the coverage estimation, values were 70%, 80% and 95%, for the mature-forest (M), the young-forest (Y) and the grassland (G), respectively. Finally, when comparing the different environments in terms of the weighted frequencies of each OTU (mycobionts and cyanobionts), the grassland (G) was the most different environment (dissimilarity of 0.91 with the cluster M-Y), while the young-forest (Y) and the mature-forest (M) were more related to each other (dissimilarity of 0.52). Moreover, the correlation of the geographical distances between the samples with respect to the genetic distances of the lichen's symbiotic components, using the Mantel test, revealed very low values ($R = 0.1607$, $P = 0.0066$ and $R = 0.2094$, $P = 0.0002$ for mycobionts and cyanobionts, respectively).

Discussion

Most of the current research on conservation of lichens biodiversity states that changes in forest cover directly affect epiphytic species. However, they could also affect the micro-

climatic conditions inside the forests, especially light and moisture, which are key influences to lichens in general, including the terricolous ones [18].

A substantial percentage of species from the native forests of the Karukinka Natural Park are endemic to the Park or to the region, and represent an important reservoir of diversity [2,8]. However, despite several efforts to protect this diversity, there are areas of the Park that present low habitat quality, either by anthropogenic (road construction, logging, introduction of exotic species, etc) or natural (fires, high winds, etc.) factors [33]. A previous baseline study conducted in different areas of the Park determined that in forest habitats, most lichens inhabit the trunks of trees (105 species, 58 %), with fewer species in the canopy (20 species, 19 %) and in the soil (25 species, 23 %). Within the latter, only 6 species of the genus *Peltigera* were reported, which are fewer species than the ones reported in the present study; even though the 18S rRNA and 28S rRNA genes provide just a conservative estimate of the *Peltigera* diversity, being slow-evolving compared with ITS, the standard species barcode for fungi [15,34]. The maximum value obtained by the Margalef index (5.7 for M) coincides with the number of species of *Peltigera* reported in this area in the previous baseline study, which agrees with the highest expected species richness in the forest contexts. Together, these data show that the sampling carried out was enough to cover most of the previously reported *Peltigera* species richness in the area and represented a substantial portion of the diversity present.

The correlation of the geographical distances and the genetic distances of the lichen's symbiotic components between the samples indicated that the habitat differences appear to be a better descriptor of the diversity of these lichens and their components than the geographic distance.

In this work, the diversity of the symbiotic components of *Peltigera* cyanolichens was quantified to determine the influence of the habitat conservation state. Considering that in microbial systematics there is not a general agreement to define the fundamental biological diversity unit [30], most of the studies based on molecular techniques adopt the concept of operational taxonomic units (OTUs) to define taxa. Therefore, this concept was applied to determine the diversity of the *Peltigera* symbiotic components based on robust phylogenetic analyses.

Our results confirm that in an undisturbed environment, such as a mature native forest, a higher diversity of lichens of the genus *Peltigera* can be found, including some specimens exclusive of this area. For example, the OTU PmO5 was associated with *P. frigida*, a cyanolichen that has mainly been reported in Tierra del Fuego Island [19]. Furthermore, the OTU PmO2 was potentially related to *P. "papuana"*, which has been only reported in a few places [36]. Strikingly, the cyanobiont OTUs related to the above mentioned mycobiont OTUs were also found exclusively in the mature-forest and associated only with one mycobiont OTU. Hence, some of the lichen fungi found in the mature native forest showed seemingly high photobiont specificity.

In the case of the young-forest, lower mycobiont and cyanobiont OTU diversities than in the mature-forest were found. Only one unique mycobiont OTU (PmO4) to this environment, and related to *P. extenuata*, was detected. Moreover, just two cyanobiont OTUs unique to this environment were found, which coincidentally were associated with the mycobiont PmO4. Members of this species of *Peltigera* have an asexual reproductive mode [9], which could be related to a low dispersal and a high specificity of the association.

In the most degraded environmental context, the grassland, a single mycobiont OTU was found (PmO1, related to the species *P. rufescens*), which was in turn the only one present in all the three environments. This species has a cosmopolitan distribution and it has been widely reported as one of the most common species of the genus *Peltigera* in the world [19,21,40]. Members of this species inhabit different environments, their presence is not necessarily linked to a wooded area, and they can often be found in grasslands, open forests (meadows) and high mountain areas [19]. In this environment, PmO1 was associated with four cyanobiont OTUs, accounting for the versatility of this organism, which could be regarded as a pioneer in the successful colonization of an environment.

It is known that loss of habitat quality can affect mating systems, causing an increase of inbreeding and an erosion of genetic diversity within populations, with differentiation between populations through stochastic processes associated with genetic drift [12,18]. The presence of unique OTUs and the greatest diversity of lichens in native forest areas highlight the importance of their preservation and thus conserve this important source of diversity.

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