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Bacterial community of reindeer lichens differs between northern and southern lichen woodlands

Marta Alonso-García and Juan Carlos Villarreal A.

Abstract: Lichens cover nearly 7% of the earth's surface, and in eastern Canada, lichen woodlands occupy over 300 000 km^2. Reindeer lichens (genus *Cladonia*) are the main component of lichen woodlands and they play a crucial role in boreal forest ecology. We study, for the first time, the bacterial community of four species of reindeer lichens from eastern North America's boreal forests. Using the 16S rRNA gene, we characterize the bacterial community of 189 lichen samples. We aim to analyse the effect of geography and host identity in the bacterial community composition and structure, verify the presence of a common core bacteria, and identify the most abundant core taxa. Our results suggest that host-lichen identity does not determine bacterial community composition and structure in reindeer lichens, but we confirm the influence of geography in shaping the diversity and abundance of bacteria associated with *Cladonia stellaris*. We also reveal that reindeer lichens share a reduced common core bacteria composed exclusively by *Alphaproteobacteria*. Northern lichen woodlands exhibit a significantly higher diversity and abundance of bacteria associated with *C. stellaris* than southern lichen woodlands do. The presence of the species *Methylorosula polaris* in the core bacteria is evident and may have a particular importance for reindeer lichens.

Key words: bacteria, *Cladonia stellaris*, geography, host identity, ITS region, multi-species model, reindeer lichens, species delimitation, symbiotic interactions, thallus morphology.

Résumé : Les lichens occupent près de 7 % de la surface terrestre, et dans l'est du Canada, les forêts à lichens occupent plus de 300 000 km^2. Les cladonies arbuscules (genus *Cladonia*) sont les principales composantes des forêts à lichens et elles jouent un rôle crucial dans l'écologie des forêts boréales. Nous étudions, pour la première fois, la communauté bactérienne de quatre espèces de cladonies arbuscules des forêts boréales de la partie est de l'Amérique du Nord. En utilisant le gène de l'ADN 16S, nous caractérisons la communauté bactérienne de 189 échantillons de lichens. Notre objectif est d'analyser l'effet de l'identité et de la provenance de l'hôte dans la composition et la structure de la communauté bactérienne, de vérifier la présence d'une bactérie de base commune et d'identifier le plus abondant taxon de base. Nos résultats suggèrent que l'identité du lichen hôte ne détermine par la composition et la structure de la communauté bactérienne dans les cladonies arbuscules, mais nous confirmons l'influence de la provenance dans la formation de la diversité et de l'abondance de la bactérie associée à la *Cladonia stellaris*. Nous révélons également que les cladonies arbuscules partagent une bactérie de base commune réduite composée exclusivement par l'alphaprotéobactérie. Les forêts à lichens nordiques présentent une diversité et une abondance sensiblement plus élevées de bactéries associées à la *C. stellaris* que les forêts à lichens australes. La présence des espèces de *Methylorosula polaris* dans la bactérie de base est évidente et elle peut avoir une importance particulière pour les cladonies arbuscules. [Traduit par la Rédaction].

Mots-clés : bactérie, *Cladonia stellaris*, géographie, identité de l'hôte, région de l'ETI, modèle plurispécifique, cladonies arbuscules, délimitation des espèces, interactions symbiotiques, morphologie du thalle.

1. Introduction

Microbiome research focuses on the interactions of microbes within a specified environment or host (Cullen et al. 2020). One of the most pressing questions in microbiome research is whether host specificity of the microbial community exists. Specificity can be considered as an interaction between microorganisms and host in which absolute exclusiveness is expressed (Bubrick et al. 1985). It should not be mistaken with host selectivity, which describes a situation where microorganisms and host interact preferentially with one another (Bubrick et al. 1985). Microorganisms display various levels of host specificity, infecting a wide range of hosts (Rahme et al. 2000; Chappell and Rausher 2016) or having strict host selectivity (e.g., in sponges (Reveillaud et al. 2014), hornworts (Bouchard et al. 2020), cetaceans (Denison et al. 2020), or humans (Pan et al. 2014)).

Despite the importance of host identity in shaping the composition and structure of a microbial community, many other biotic or abiotic factors can determine the microbiota. Among the abiotic factors, geography and environmental conditions are probably the best studied (Rothschild et al. 2018; Zheng and Gong 2019; Sepulveda and Moeller 2020). Regarding host-related factors, physiology (Reveillaud et al. 2014; Denison et al. 2020), morphology (Pearce et al. 2017; Morrissey et al. 2019), or genotype

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(Wagner et al. 2016) are those that have, so far, received more attention. Likewise, stochastic colonization and microbial interactions (Hassani et al. 2018) can also contribute to community composition and structure.

Another key aspect to consider in microbiome research is the prevalence and frequency of microorganisms in the host, namely, the core microbiome. The core microbiome is defined as a group of microbial taxa that occur with hosts above a particular occupancy frequency threshold (Risely 2020), often between 30% (Ainsworth et al. 2015) and 95% (Huse et al. 2012) (common core). The major motivation for identifying a universal common core is to find a component of the microbiome that, due to the higher prevalence, may have a particular positive effect on the host.

Among non-model organisms, lichens are the symbiotic organism "par excellence", because of a partnership between fungi (one to several species), green algae and (or) cyanobacteria, and numerous bacteria in a multi-species symbiosis (Aschenbrenner et al. 2016; Lavoie et al. 2020) or a mini-ecosystem (Hawksworth and Grube 2020). While the interactions between the fungus and photobiont have been intensively studied, the role of bacteria in the symbiosis is still in its infancy (Grube et al. 2015). Genomic exploration of lichen-associated microbes has revealed an unexpected diversity of bacteria, the majority belonging to *Alphaproteobacteria* (Cardinale et al. 2008; Grube and Berg 2009; Hodkinson and Lutzoni 2009; Bates et al. 2011; Printzen et al. 2012). The bacterial community of lichens contributes to essential functions of the host (nutrient supply, resistance against biotic and abiotic factors, growth support, detoxification of metabolites, or provision of vitamin B_{12}) (Grube et al. 2015). Lichen bacterial community can be determined by different factors, such as host identity (Bates et al. 2011; Sierra et al. 2020), photoautotrophic symbiont (Hodkinson et al. 2012), thallus conditions (Mushegian et al. 2011; Cardinale et al. 2012*b*; Noh et al. 2020) and growth form (Park et al. 2016), substrate type (Park et al. 2016), habitat (Cardinale et al. 2012*a*), and (or) geography (Hodkinson and Lutzoni 2009; Aschenbrenner et al. 2014). To date, studies on lichen bacterial community have been mainly carried out with *Lobaria pulmonaria* (L.) Hoffm. (Cardinale et al. 2012*a*; Aschenbrenner et al. 2014; Grimm et al. 2021), *Cetraria aculeata* (Schreber) Fr. (Printzen et al. 2012), *Cladonia arbuscula* (Wallr.) Rabenh. (Cardinale et al. 2008), or *Cladonia squamosa* Hoffm. (Noh et al. 2020). However, there is still a large and unexplored microbial diversity in other groups of lichens, such as those from northern ecosystems.

Reindeer lichens (Ahti 1961) are terricolous lichens that have adapted to boreal forests better than almost all other lichens (Athukorala et al. 2016). Species such as *Cladonia mitis* Sandst., *Cladonia rangiferina* (L.) F.H.Wigg., *Cladonia stellaris* (Opiz) Pouzar & Vězda, and *Cladonia stygia* (Fr.) Ruoss are essential components of boreal forest ecosystems, and in winter, they represent the most important food source for reindeer (*Rangifer tarandus* Linnaeus, 1758) and caribou (*R. tarandus caribou* Gmelin, 1788) (Skogland 1984; Svihus and Holand 2000; Thompson et al. 2015). Within the boreal biome, lichens are particularly dominant in lichen woodlands (LWs), a belt between the closed-canopy boreal forest to the south and the forest tundra to the north, mostly above the 50th parallel (Payette 1992; Johnson and Miyanishi 1999). In eastern North America, a remnant of LW is located 500 km south of its usual distribution range, in the Parc des Grands-Jardins (PNGJ) (Jasinski and Payette 2005).

Based on the importance of lichens in northern ecosystems as well as their utility as multi-species model to study symbiotic relationships, here we investigate the bacterial community of four lichen species in the boreal forest. More specifically, we (*i*) analyse host selectivity of bacteria associated with reindeer lichens, (*ii*) assess the influence of geography in composition and structure of the bacterial community of *C. stellaris* from LWs, and (*iii*) verify the presence of a common core bacteria in reindeer lichens. To achieve our goals, we used four reindeer lichen species (*C. mitis*, *C. rangiferina*, *C. stellaris*, and *C. stygia*), and we identified the bacterial community by using 16S ribosomal RNA (rRNA) metabarcoding. Bacterial host selectivity was studied based on the widely accepted morphological definition of each lichen species. Molecular species delimitation based on one internal transcribed spacer (ITS) locus was also performed, and the results were compared. The influence of geography on the bacterial community was carried out including a single species (*C. stellaris*) and a single ecosystem (LWs). Finally, we probed for bacterial taxa occurring with reindeer lichens above a particular occupancy frequency threshold and whose presence could be interesting for the host–bacteria symbiotic relationship.

2. Materials and methods

2.1. Sample collections and processing

We studied four species of reindeer lichens: *C. mitis*, *C. rangiferina*, *C. stellaris*, and *C. stygia* (Ahti 1961) (Fig. 1). We collected 192 samples along a north–south gradient in the province of Quebec (Canada) (Fig. 2) to have a broad representation of reindeer lichens from eastern North America. We gathered samples from six bioclimatic domains: shrub tundra, forest tundra, LW, closed-crown forest, balsam fir – white birch, and balsam fir – yellow birch forests. We selected three sites per domain (Fig. 2), and we collected between five and 10 samples per site, depending on availability (supplementary S1[1]). The minimum distance between sites was at least 25 km, and the maximum was never larger than 200 km. From the tundra, eight samples were included, due to difficulty to collect fresh samples in the logistically complex tundral regions of Quebec. To achieve our second objective, we intensified sampling efforts in LWs in northern (Kuujjuarapik-Whapmagoostui) and southern (PNGJ) Quebec, gathering 28 and 58 samples, respectively. The presence of a southern LW in PNGJ (Jasinski and Payette 2005) makes this region an ideal setting to explore the effect of geography. It allows us to carry out the comparison under the same environmental conditions (two LWs) in geographically distant areas (northern and southern Quebec).

For each sample, the last 2 cm of the apical parts of thallus lichen were selected and taken with steel forceps. Thallus fragments were placed into Eppendorf tubes and stored at –20 °C immediately after collection. No washing treatment was applied to the lichens. Steel micro forceps were sterilized with 70% ethanol between collections.

The identity of the samples was verified using regional taxonomic publications (Brodo et al. 2001). Herbarium vouchers were then deposited in the Louis-Marie Herbarium (QFA), Laval University. Supplementary S1[1] contains vouchers information including collector, collection number, species identity, locality, vegetation zone (arctic, boreal, or temperate) (Rowe 1972), bioclimatic domain (shrub tundra, forest tundra, LW, closed-crown forest, balsam fir – white birch, balsam fir – yellow birch) (Rowe 1972), altitude, type of genetic data generated, GenBank accession numbers, and samples included in each dataset.

2.2. DNA extraction, PCR amplification, and sequencing

Lichen genomic DNA was extracted following an established potassium chloride protocol (Park et al. 2014). The internal transcribed spacers 1 and 2 and the 5.8S rRNA (hereinafter ITS) of the nuclear ribosomal DNA (rDNA) were selected to perform molecular species delimitation of lichens. Following Steinová et al. (2013) protocols, we successfully amplified and sequenced 104 lichen

[1]Supplementary data are available with the article at https://doi.org/10.1139/cjfr-2021-0272, and consist of the datasets generated and analysed during the current study, which are available in the NCBI SRA archive under Bioprojects PRJNA593044 and PRJNA687262.

Fig. 1. Reindeer lichen species used in the study: (A) *Cladonia mitis*, (B) *Cladonia stellaris*, (C) *Cladonia rangiferina*, and (D) *Cladonia stygia*. Photos by Troy McMullin (Canadian Museum of Nature – Research and Collections). [Colour online.]

samples, allowing for a broad representation of the four morphological species from the six bioclimatic domain (supplementary S1[1]).

To identify bacteria associated with our samples, the V3–V4 region of the 16S rRNA gene was amplified for 189 lichen samples (supplementary S1[1]), following an amplicon sequencing protocol developed at Laval University (Vincent et al. 2017). For the first PCR, the locus-specific primers BactV3-V4-F (341F) and BactV3-V4-R (805R) were selected from Herlemann et al. (2011) and were modified to include Illumina TruSeq sequencing primers on their 5′ ends. The second PCR introduced indexes and Illumina adapters used in library construction. The libraries were pooled using an equimolar ratio, quantified, and sequenced on an Illumina MiSeq 300 bp paired-end run at the Plateforme d'analyses génomiques at the Institut de biologie intégrative et des systèmes (Université Laval, Québec, Canada).

2.3. Molecular delimitation of reindeer lichen species

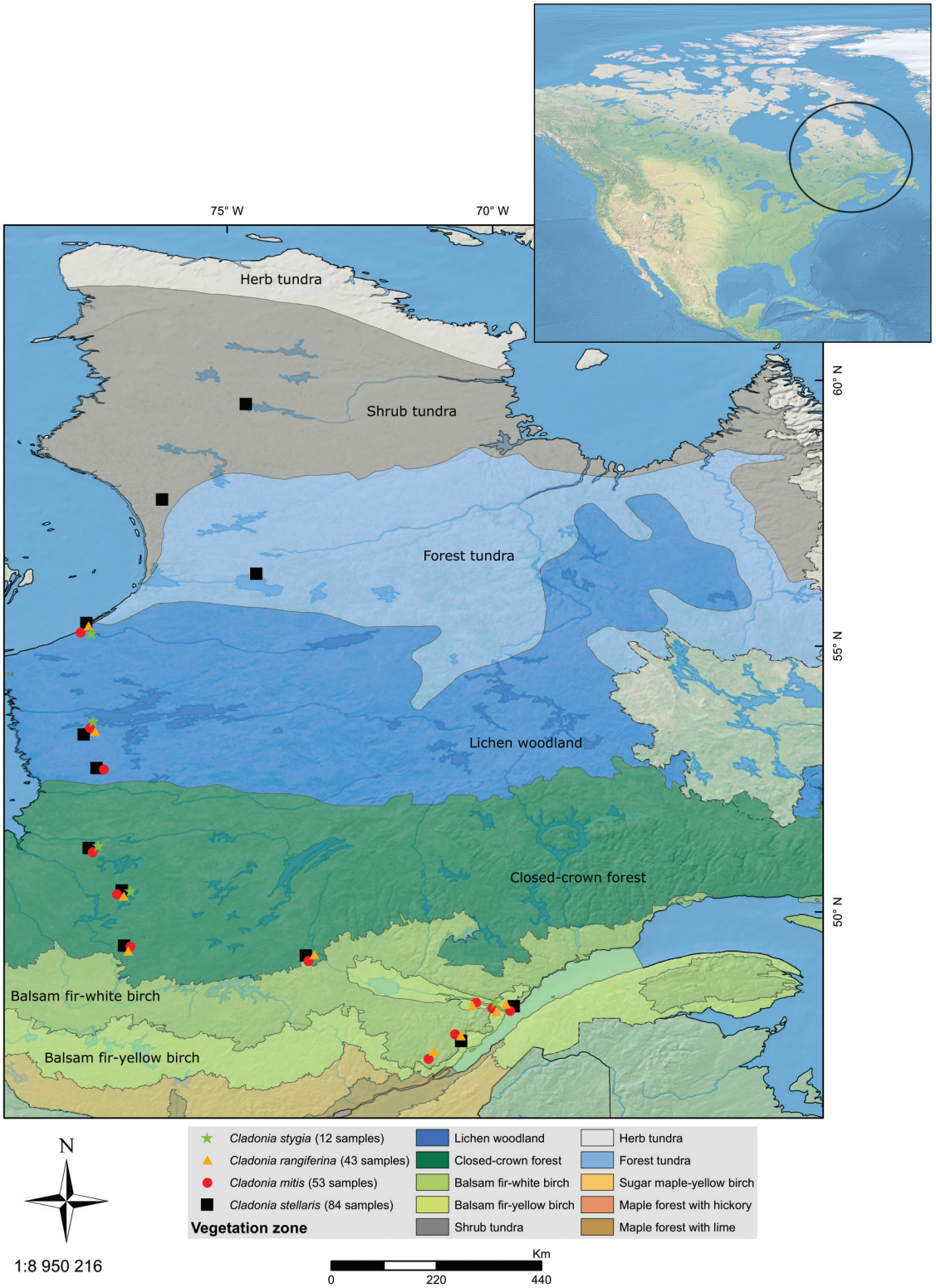
We conducted Bayesian inferences (BI) using the program MrBayes version 3.2 (Ronquist et al. 2012) on the ITS dataset, including our 104 sequences plus 22 from GenBank (supplementary S1[1]). *Cladonia wainioi* Savicz was selected as outgroup (Stenroos et al. 2019). We tested the best-fit substitution model using MrModelTest (Nylander 2004) using the Akaike information criterion. The *allcomat* option was included to have a binary tree, required for the next set of analyses.

We tested the Poisson tree processes (PTP) model of species delimitation (Zhang et al. 2013) for the BI tree. A Bayesian implementation of the PTP model (bPTP) (Zhang et al. 2013) was performed on the online server https://species.h-its.org/ using the BI tree. We ran 500 000 generations, then assessed convergence visually. Additionally, we applied the recently introduced multirate PTP (mPTP) (Kapli et al. 2017) method, an improved version of the PTP for single-locus species delimitation, which has been shown to be consistent and very effective for species delimitation in datasets with uneven sampling (Blair and Bryson 2017), and it has also been successfully applied to lichens (Kistenich et al. 2019). Using the stand-alone mPTP software (version 0.2.4) (Kapli et al. 2017), we performed the analyses on the BI tree. We first calculated the correct minimum branch length threshold (*–minbr_auto*). Then, we executed a maximum-likelihood (ML) species delimitation inference (*–multi –outgroup_crop*). To assess the confidence of the ML delimitation scheme, we conducted four Markov chain Monte Carlo (MCMC) runs for 20 million generations, sampling every 5000. The first two million generations were discarded as burn-in, and analyses started with a random delimitation (*–mcmc_startrandom*). We compared results among MCMC runs to assess congruence.

2.4. Taxonomic assignment of bacterial sequences

We used the DADA2 workflow to assign amplicon sequence variants (ASVs; Callahan et al. 2017) in R (Callahan et al. 2016) to 189 lichen samples. We trimmed and filtered the raw reads,

Fig. 2. Map of eastern North America with sampling localities of the 192 reindeer lichen specimens included in this study. Four species of *Cladonia* are represented by different symbols, red circles for *C. mitis*, orange triangles for *C. rangiferina*, black squares for *C. stellaris*, and green stars for *C. stygia*. Bioclimatic domains highlighted following Rowe (1972). All of the data are defined to be in the NAD83 projection and the UTM coordinate systems. Made with Natural Earth. Free vector and raster map data at https://www.naturalearthdata.com. [Colour online.]



keeping only those with quality scores higher than 25. We dereplicated (*derepFastq*) all reads, estimated their error rates (*learnErrors*), and denoised them (*dada*). Then, forward and reverse reads were merged (*mergePairs*). All merged sequences with less than 430 bp, and more than 450 bp were removed, and chimeras were excluded. We assigned taxonomy based on the SILVA 138 database (McLaren 2020) with minimum bootstrap set to 80.

The phangorn R package (Schliep 2011; Schliep et al. 2017) was used to build a phylogenetic tree, used in downstream analyses and to estimate phylogenetic distances between microbial communities. We first built a neighbor-joining tree, and then fit a GTR+Γ+I (generalized time-reversible with gamma rate variation and invariant sites) ML tree using the neighbor-joining tree as a starting point.

We synthesized all the data generated (ASV table, sample data, taxonomy table, phylogenetic tree, and environmental variables; supplementary S1[1]) into a single phyloseq object with the R package phyloseq (McMurdie and Holmes 2013). ASVs corresponding to chloroplast and mitochondria were removed from the dataset. We also removed phyla with less than 10 corresponding ASVs in all samples combined, as well as bacteria for which no phylum could be assigned. We determined prevalence (fraction of samples in which an ASV occurs) and used it to create a second phyloseq object including only the ASVs occurring in at least 5% of the samples. This prevalence filter avoided spending much time analysing taxa that were seen only rarely among samples and it was a useful filter of taxa that are actually just artifacts (Callahan et al. 2016).

2.5. Characterization of bacterial communities in reindeer lichens

We applied diversity and composition analyses to two sets of data: (*i*) the host-species dataset to analyse bacteria host selectivity in reindeer lichens, consisting of 101 samples (objective *i*), and (*ii*) the LWs dataset to assess the influence of geography in *C. stellaris*, consisting of 47 samples (objective *ii*) (supplementary S1[1]). Statistical analyses were carried out to test whether geography and host identity were significant predictors of community composition for bacteria. With the R package phyloseq (McMurdie and Holmes 2013), we estimated the number of observed ASVs and the alpha-diversity within each sample based on Shannon and Simpson effective indices. The Shannon index accounts for species richness and its evenness or equitability, whereas the Simpson index estimates dominance of the species. Shapiro–Wilk tests indicated if the data were normally distributed. We then used parametric (ANOVA) or nonparametric (Kruskal–Wallis and Mann–Whitney *U* tests) statistics methods to test for significant differences. To evaluate beta-diversity, that is, the diversity between samples, the weighted UniFrac distance matrix was calculated. Unlike other metrics, UniFrac takes into account phylogenetic information. Principal coordinates analysis (PCoA) and double principal coordinates analysis (DPCoA) based on the weighted UniFrac distance matrix were plotted. To determine whether bacterial communities significantly differ among lichen species, PERMANOVA tests and pairwise comparison were conducted (2000 permutations) using the adonis and pairwise.adonis (Martinez Arbizu 2020) functions in the vegan R package (Oksanen et al. 2020). We transformed ASV counts per sample into relative abundance and compared it among species, and LWs to assess the structure of the bacterial community. To identify specific ASVs that show differential abundance among taxa, the R package DESeq2 (Love et al. 2014) was used. We removed samples with less than 1000 reads and applied a normalized logarithmic transformation (rlog) on the ASVs. We estimated logarithmic fold change and dispersion for each ASV. We obtained an adjusted *p* value (*padj*) to correct for false positives (false discovery rate) using the Benjamini–Hochberg correction. These ASVs sequences were aligned to sequences in the NCBI database 16S ribosomal RNA (Bacteria and Archaea) using Megablast optimized for highly similar sequences. The identifications considered successful were those with over 97% similarity (Stackebrandt and Goebel 1994). Below this percentage, we treated the sequences as relatives.

2.6. Detection of core bacterial members in reindeer lichens

The core bacteria was identified with the microbiome R package (Lahti and Shetty 2012). Due to the lack of consensus about a fixed threshold (Risely 2020), we considered as core bacteria any taxon with a prevalence higher than 0.50, 0.75, or 0.90 (Jorge et al. 2020). We included 189 lichen samples in the analysis. We pruned out the phyloseq object to retain those samples with more than 1000 reads; in the end 153 samples were incorporated. We detected the core bacteria with the function (*core_members*) and estimated the total core abundance in each sample (*sample_sums*). A heatmap was elaborated to visualize the results. To identify the core taxa at species level, ASV sequences were aligned to sequences in the NCBI database 16S ribosomal RNA (Bacteria and Archaea) using Megablast optimize for highly similar sequences and retaining those with BLAST hits over 97% identity. Bacterial sequences with an identity below 97% were considered as relatives. Due to the predominance of *C. stellaris* over the remaining reindeer lichens in eastern Canada, we carried out the same analyses to predict the core bacteria of this species.

3. Results

3.1. Identity of reindeer lichen species

To analyse host selectivity, we grouped lichen samples based on morphology and based on the phylogeny of the ITS locus. According to our morphological identification, we had 53 samples belonging to *C. mitis*, 43 to *C. rangiferina*, 84 to *C. stellaris*, and 12 to *C. stygia*.

Regarding the molecular species delimitation, we successfully amplified the ITS locus of 104 samples representing four morphological species of *Cladonia*: 40 individuals of *C. mitis*, 34 of *C. rangiferina*, 21 of *C. stellaris*, and nine of *C. stygia*. Twenty-two sequences from GenBank were added to the 104 generated sequences (supplementary S2[1]). The BI phylogenetic tree supported (PP $\geq$ 0.95) a clade including all the reindeer lichens (supplementary S3[1]). *Cladonia stellaris* split into two groups: a supported clade (clade *C. stellaris* species 1; supplementary S3[1]) and an unsupported clade (clade *C. stellaris* species 2; supplementary S3[1]), with the only exception being a single specimen from GenBank (accession No. KP001212 from Newfoundland). Collections of *C. mitis*, *C. rangiferina*, and *C. stygia* clustered in a supported clade split into six unsupported subclades: four including *C. mitis* and two grouping *C. rangiferina* and *C. stygia*.

The MCMC chains for the bPTP species delimitation method did not converge, thus the model was not further explored. The mPTP method inferred 10 species, with strongly supported results (average support values over 0.91). *Cladonia mitis* was divided into four taxa; *C. stellaris* split into three taxa, one of them including exclusively a single specimen from GenBank; finally, individuals belonging to *C. rangiferina* and *C. stygia* were merged but split into two taxa (supplementary S4[1]). Supplementary S3[1] shows the BI phylogenetic tree with the species identified by the mPTP method.

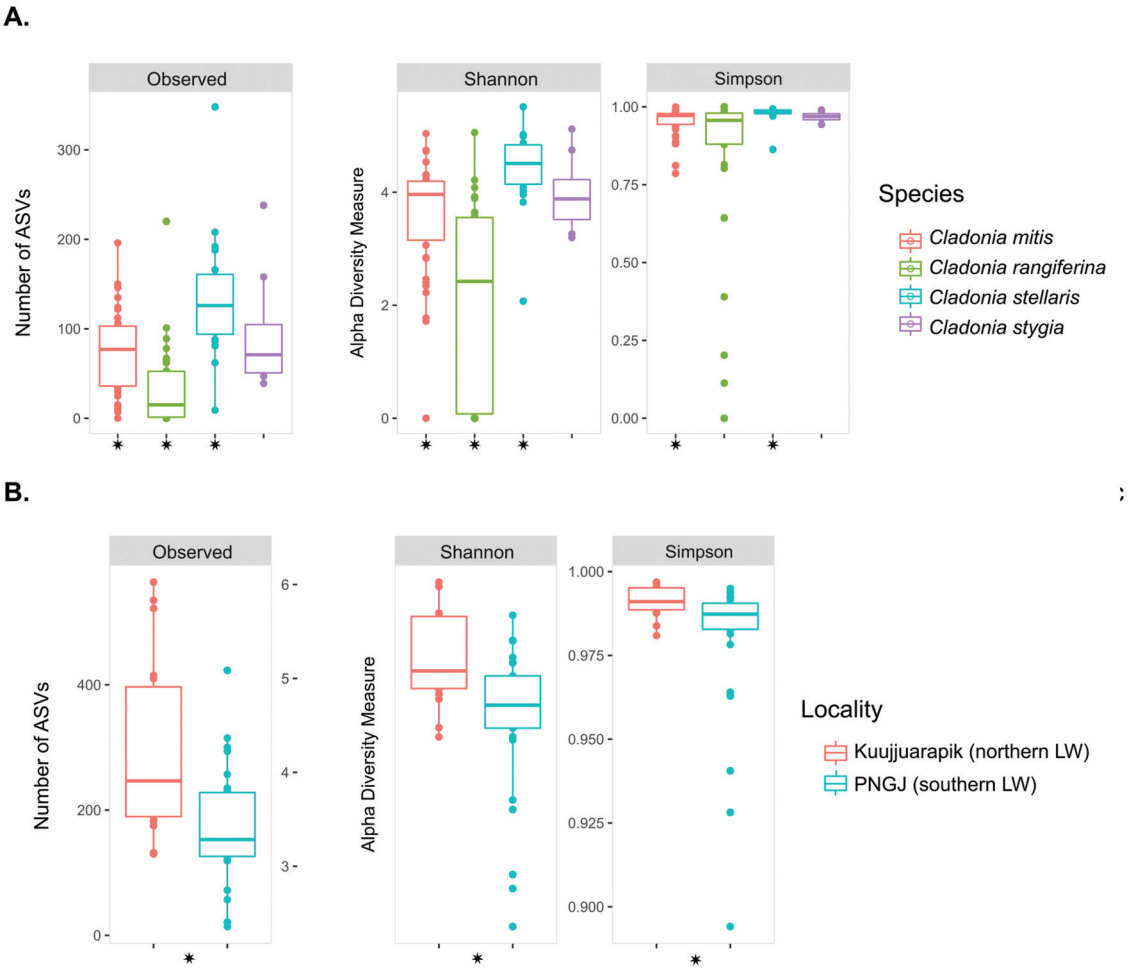
3.2. Dominance of *Proteobacteria* in reindeer lichens

A total of 6 204 737 raw reads from 16S rRNA were identified from 189 samples of reindeer lichens (supplementary S5[1]). After quality trimming, 817 688 reads were kept. High-quality reads were assigned to 12 917 ASVs. Once we removed chloroplast and mitochondrial ASVs and phyla with less than 10 ASVs, we retained 10 969 ASVs. The prevalence filter (ASVs occurring in at least 5% of the samples) preserved 580 ASVs in 101 samples (host-species dataset), and 1130 ASVs in 47 samples (LWs dataset). All the ASVs could be assigned at phylum level. The ASVs corresponded to *Acidobacteriota*, *Cyanobacteria*, *Candidatus* Eremiobacterota (candidate division WPS-2), *Planctomycetota*, *Proteobacteria*, and *Verrucomicrobiota*. Most of the sequences belonged to *Proteobacteria* (ca. 80%), followed by *Ca.* Eremiobacterota. Supplementary S6[1] displays the number of ASVs detected for each dataset at phylum level. Bacteria relative abundance of each lichen sample is showed in supplementary S7[1].

3.3. Host selectivity in reindeer lichens

The bacterial alpha diversity within morphological species (Fig. 3A) was always significantly different ($p < 0.01$) in the number of observed ASVs and Shannon index (supplementary S8[1]),

Fig. 3. Richness estimate values (number of observed amplicon sequence variants, ASVs) and alpha diversity indices (Shannon and Simpson) for reindeer lichens bacterial communities. (A) Diversity values of reindeer lichens species. Asterisks indicate significant differences ($p < 0.01$) between *C. mitis* and *C. rangiferina*, *C. mitis* and *C. stellaris*, *C. rangiferina* and *C. stellaris*, *C. rangiferina* and *C. stygia* (observed ASVs and Shannon index), and *C. mitis* and *C. stellaris* (Simpson index). (B) Diversity values of *Cladonia stellaris* from lichen woodlands (LWs) in northern and southern Quebec (LWs dataset). Asterisks indicate significant differences ($p < 0.01$) between LWs. [Colour online.]



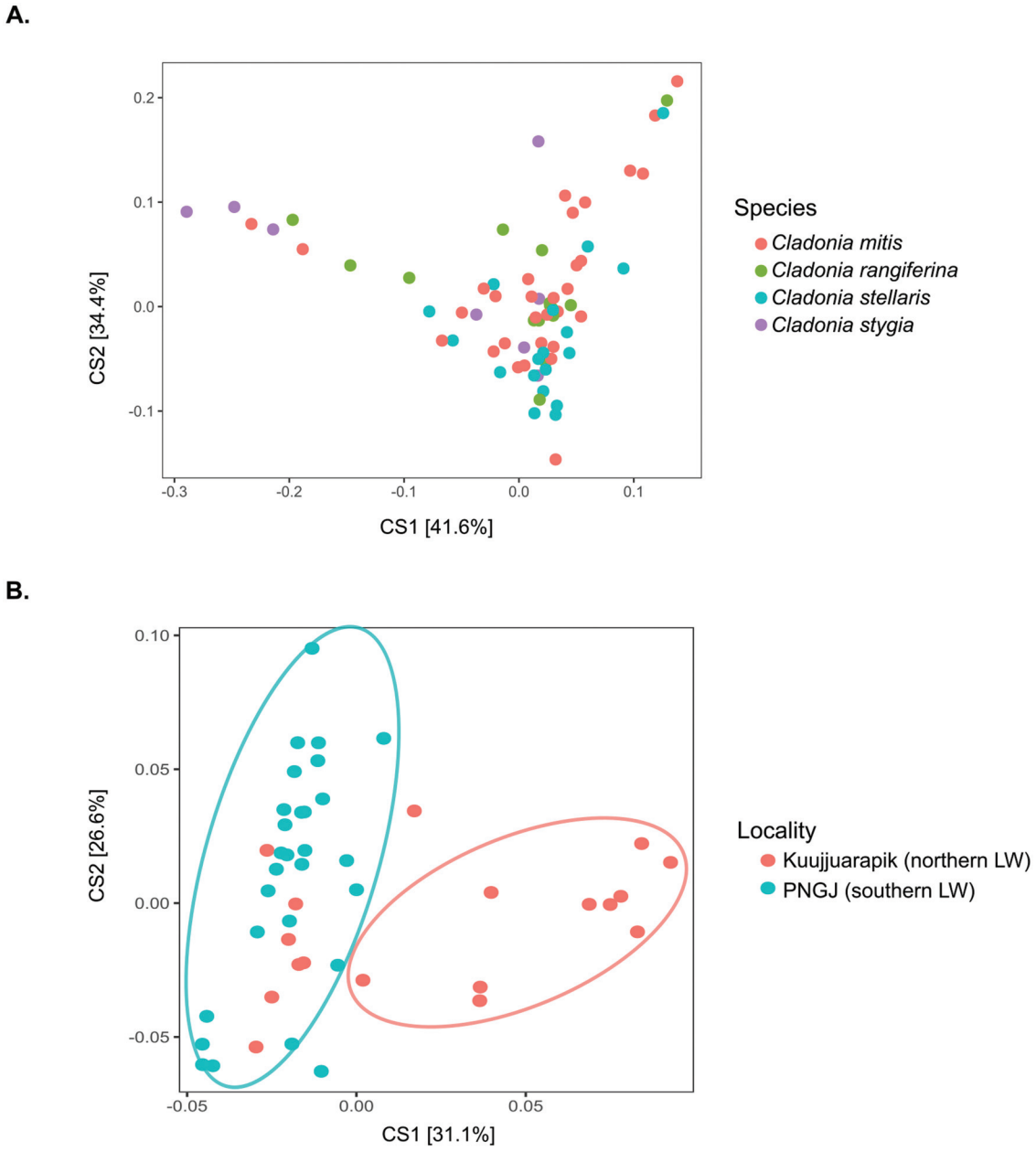
except between *C. stygia* with *C. mitis* or *C. stellaris* (supplementary S8[1]). Significant differences in diversity for the Simpson index ($p < 0.01$) were detected exclusively between *C. mitis* and *C. stellaris* (supplementary S8[1]). Different results obtained by Shannon and Simpson were expected because Simpson index considers exclusively dominance of the species, but not species richness (as Shannon index). In the PCoA, axes 1 and 2 explained 59.2% and 20.2% of the total variation among samples, respectively (supplementary S9A[1]). In the DPCoA, CS1 explained 41.6% and CS2 explained 34.4% of the total variation (Fig. 4A). Bacterial communities of reindeer lichens were not grouped according to host species (supplementary S9A and S10A[1]), although PERMANOVA tests found a significant difference ($p = 0.018$) between the bacterial community composition of *C. stellaris* and *C. stygia* (supplementary S11[1]). When considering species delimited by the mPTP method applied to the ITS locus, alpha diversity was significantly different between *C. stellaris* species 2 and *C. rangiferina* and *C. stygia* species 2 in observed ASVs and Shannon index ($p < 0.01$) (supplementary S8 and S10[1]). No significant differences in diversity were detected for the Simpson index ($p > 0.01$) (supplementary S8[1]). PERMANOVA tests found no significant association of the bacterial communities according to molecular species.

Proteobacteria were dominant across all reindeer lichens (supplementary S7A[1]), although there were some exceptions. Two lichen taxa, one identified as *C. mitis* (*Alonso 422*) and the other one as *C. rangiferina* (*Alonso 433*) (supplementary S1[1] for voucher information), harboured mostly *Ca.* Eremiobacterota. Two other lichens belonging to *C. rangiferina* included only *Cyanobacteria* (*Alonso 423*) or mostly *Planctomycetota* (*Alonso 475*). DESeq2 analysis identified ASVs that displayed a significant change of abundance (*padj* <0.05) compared to host lichen species (supplementary S12A and S12B[1]). Bacterial communities of reindeer lichens exhibited differences in abundance for exclusively three ASVs (supplementary S12A[1]), two members of *Alphaproteobacteria* and one of *Acidobacteria*. For the eight molecularly delimitated species (mPTP method), four ASVs were detected, belonging to *Alphaproteobacteria* (3) and *Planctomycetes* (supplementary S12B[1]).

3.4. Effect of geography in the bacterial community of *C. stellaris*

Lichens from northern and southern LWs exhibit significant differences ($p < 0.01$) in bacterial diversity (Fig. 3B) (supplementary S13[1]). Number of observed ASVs as well as Shannon and Simpson indexes reported higher diversity for northern samples (Fig. 3B). The two first axes of the PCoA explained 28.1% and 21.8% of the total variation, respectively (supplementary S9B[1]), whereas axes 1 and 2 of DPCoA explained 31.1% and 26.6%, respectively (Fig. 4B). Both ordination methods showed two different bacteria groups based on the latitude, northern and southern LWs (supplementary S9B and

Fig. 4. Double principal coordinates analysis (DPCoA) of bacterial community composition based on amplicon sequence variants (ASVs) in (A) reindeer lichens and (B) *Cladonia stellaris* from lichen woodlands (LWs) in northern and southern Quebec. Bacterial communities are not grouped by host species. Two clusters are differentiated between northern and southern LWs ($p < 0.01$). [Colour online.]

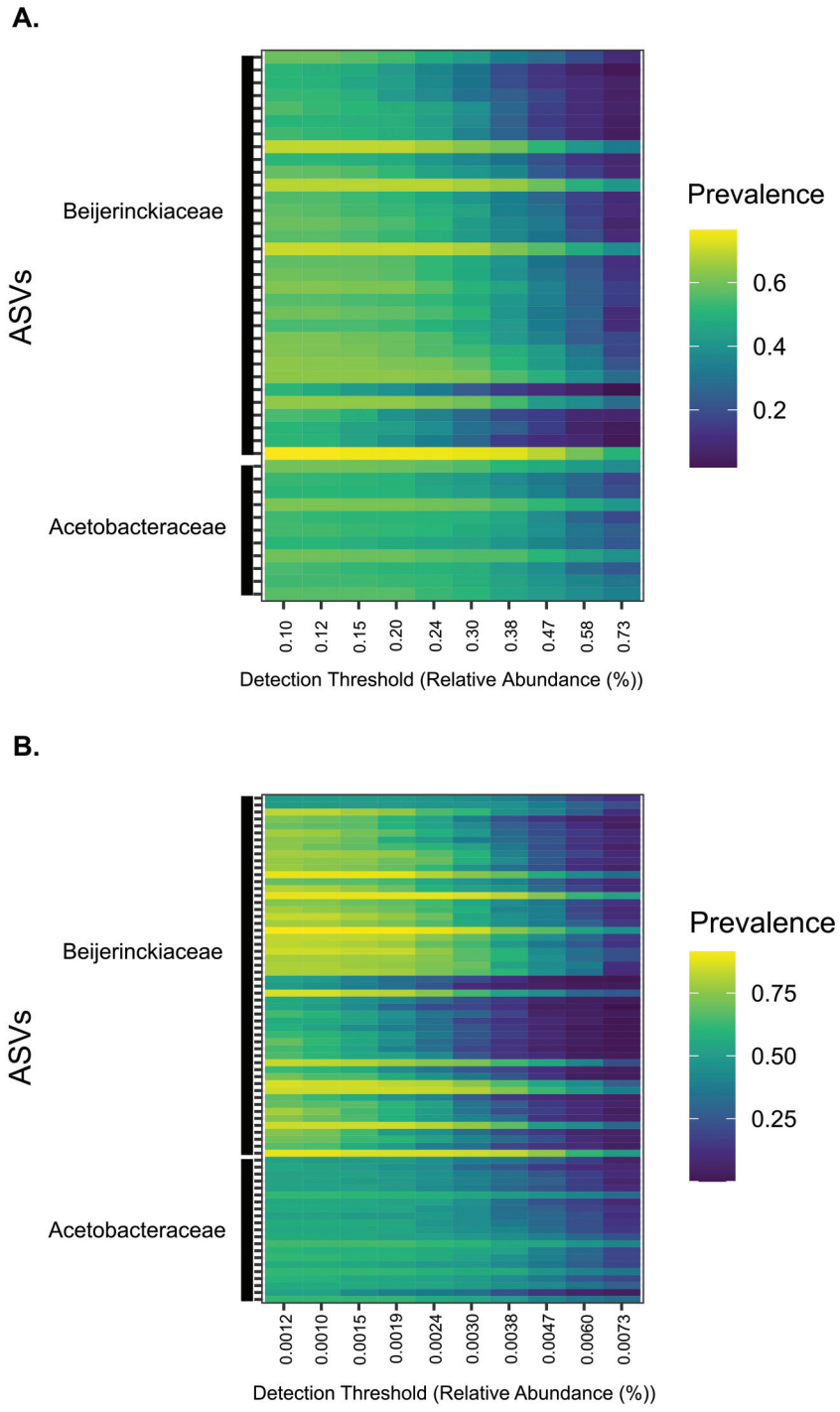


S10B[1]), and PERMANOVA test confirmed this association ($p < 0.01$) (supplementary S14[1]). Relative abundance analysis pointed out the higher abundance of *Ca.* Eremiobacterota in northern LWs (supplementary S7B[1]), except for in five samples where this phylum was nearly absent (samples *Alonso 296*, *312*, *315*, *326,* and *354* in supplementary S7B and in S1[1] for voucher information). A total of 63 ASVs were significantly different in abundance between northern and southern LWs (supplementary S12C and S15A[1]). They belonged to families *Acetobacteraceae*, *Caulobacteraceae*, *Isosphaeraceae*, and the phylum *Ca.* Eremiobacterota. The genus *Endobacter* (*Acetobacteraceae*) was the only group significantly more abundant in the south (PNGJ) than in the north (Kuujjuarapik) (supplementary S15B[1]). According to the BLAST alignment (supplementary S16[1]), their closest known relative was *Gluconacetobacter tumulioli*, with about 95.3% identity (supplementary S12C[1]). The remaining ASVs were always more abundant in LWs in northern Quebec (Kuujjuarapik) (supplementary S15B[1]). According to the BLAST algorithm, they are related to *Rhodopila globiformis* (*Acetobacteraceae*) (96.8% identity) and *Tundrisphaera lichenicola* (*Isosphaeraceae*) (94.9% identity) (supplementary S12C[1]). Additionally, 15 ASVs were identified as relatives of the bacterium species *Desulfofundulus thermocisternus* (*Ca.* Eremiobacterota), although its percentage of identity is below 85% (supplementary S12C[1]). Finally, the family *Caulobacteraceae* seemed to be represented by species related to *Brevundimonas vesicularis* (96.6% identity) (supplementary S12C[1]).

3.5. Reduced core bacteria in reindeer lichens

Forty-five ASVs were present in 153 reindeer lichens based on a 0.50 prevalence cut-off (supplementary S17A[1]), representing a minor part (ca. 5%) of the total number of ASVs (863 ASVs). All

Fig. 5. Common core bacteria of reindeer lichens at family as a function of the abundance threshold for (A) reindeer lichens and (B) *Cladonia stellaris*, with prevalence above 0.50. The *x*-axis represents the detection thresholds (indicates as relative abundance) from lower (left) to higher (right) abundance values. Colour shading indicates the prevalence of each bacterial family among samples for each abundance threshold. As we increase the detection threshold, the prevalence decreases. [Colour online.]



of them belonged to *Proteobacteria*; 32 were identified as genus *Methylocella* (family *Beijerinckiaceae*). A single ASV from *Beijerinckiaceae* as well as 12 members of family *Acetobacteraceae* were not assigned at genus level. Using the prevalence threshold of 0.75, one ASV (*Methylocella*) was detected (supplementary S17B[1]), whereas no ASVs occurred in all reindeer lichens at a prevalence of 0.90. The relative abundance of the common core members was variable among lichen samples. For a prevalence of 0.50, it ranged from 2% in two samples of *C. stellaris* (supplementary S18[1]) to 52% in one sample of *C. mitis* (supplementary S18[1]). Using 0.75 as prevalence level, the highest relative abundance of the single ASV was 13% in one sample of *C. mitis*. Thirty-six of the 153 lichens lack this ASV (0% of relative abundance) (supplementary S18[1]). The heatmap indicates the prevalence of each ASV for each abundance threshold (Fig. 5A). Four *Methylocella* ASVs presented the highest prevalence (yellow ASVs). The results derived from the BLAST alignment are shown in supplementary S19[1]. The potential identity of each ASV based on NCBI database is included in supplementary S17[1]. *Methylorosula polaris*

(identity over 97.7%) and sequences belonging to order *Rhodospirillales* (identity below 97%) were found in the common core bacteria.

A total of 81 samples of *C. stellaris* shared 87, 34, and 1 ASVs for a prevalence of 0.50, 0.75, and 0.90, respectively (supplementary S20[1]). The heatmap shows higher levels of prevalence for members of family *Beijerinckiaceae* than for the *Acetobacteraceae* (Fig. 5B). With the best BLAST hits, the common core bacteria were composed of 55 taxa of the bacteria species *Methylorosula polaris* (identity over 97.7%) and 30 taxa closely related to *Granulibacter bethesdensis* (identity around 96.6%) (supplementary S21[1]). Two relatives of *Methylocystis bryophila* were also included in the common core of *C. stellaris* with a percentage of identity of ca. 94%. Venn diagrams display the overlap between the common core bacteria of reindeer lichens (47 ASVs) and that of *C. stellaris* (supplementary S22[1]). All the ASVs associated with reindeer lichens were included in the common core of *C. stellaris*.

4. Discussion

We present the first characterization of the bacterial community in reindeer lichens from eastern North America. We analysed the influence of two factors (geography and host identity) in shaping the bacterial community composition and structure, we verified the presence of a common core bacteria, and we identified the most abundant core taxa in all reindeer lichens, with an emphasis on *C. stellaris*. Our results showed a dominance of *Alphaproteobacteria* in reindeer lichens. We found significant differences in diversity and abundance of bacteria associated with *C. stellaris* from northern or southern LWs, and we also revealed that reindeer lichens share a reduced common core bacteria composed exclusively of *Alphaproteobacteria*. The bacterial community of reindeer lichens does not group together by host species, based on neither morphology nor molecular delimitation.

4.1. *Proteobacteria* are dominant also in reindeer lichens

Lichens contain a high diversity of bacteria, the majority belonging to *Alphaproteobacteria* (Cardinale et al. 2008; Grube and Berg 2009; Hodkinson and Lutzoni 2009; Bates et al. 2011; Printzen et al. 2012). In reindeer lichens, this pattern is maintained: we found a dominance of the class *Alphaproteobacteria*, followed by the phylum *Ca.* Eremiobacterota. Our results also agreed with three previous studies of the genus *Cladonia*. Cardinale et al. (2006) isolated three species of *Proteobacteria*, *Inquilinus limosus* (family *Rhodospirillaceae*, *Proteobacteria*), *Burkholderia glathei*, and *Burkholderia sordidicola* (family *Burkholderiaceae*), as well as the species *Paenibacillus pabuli* (family *Paenibacillaceae*, phylum *Firmicutes*) from the lichen *C. rangiferina*. The bacterial community of *C. arbuscula* also showed a greater abundance of *Alphaproteobacteria* based on FISH (fluorescence in situ hybridization) experiments (Cardinale et al. 2008). More recently, (Noh et al. 2020) compared the microbiota of *C. squamosa* along the thallus and illustrated the dominance of *Alphaproteobacteria*, *Acidobacteria*, and *Ca.* Eremiobacterota.

4.2. Absence of host selectivity in reindeer lichens

Four species were considered to analyse host selectivity of the bacterial community in reindeer lichens. We also included a molecular species delimitation based on the ITS molecular marker, comparing mPTP and bPTP methods. Among the four morphological reindeer lichen species included here, the selected mPTP method identified eight species, four *C. mitis*, two *C. stellaris*, and two *C. rangiferina* and *C. stygia* grouped together. The analyses of the bacterial community were applied to molecular and morphological species. However, our results must be interpreted with caution. Species delimitation in *Cladonia* is challenging and relationships among species are not resolved (Pino-Bodas et al. 2013; Kanz et al. 2015; Athukorala et al. 2016; Stenroos et al. 2019). In addition, single-locus species delimitation methods have limitations and they are far from perfect (Blair and Bryson 2017; Dellicour and Flot 2018). We are aware, therefore, that our molecular species delimitations might change if the number of loci increases. We have prioritized the discussion and illustrations of the morphological species delimitation, but we included the molecular results as supplementary data[1].

Bacterial diversity within each sample differs between morphological species, but it hardly varies between the eight molecular species. Sierra et al. (2020) compared bacterial community in seven lichen genera, and showed differences in alpha diversity exclusively between *Usnea* and *Hypotrachyna*, although there was no information available at species level.

The bacterial community of reindeer lichens does not cluster together by host species, although values of beta diversity are significant between *C. stellaris* and *C. stygia*. Our results differ from previous studies that demonstrated that the bacterial community in lichens are host-specific (e.g., Grube et al. 2009; Bates et al. 2011; Sierra et al. 2020). Grube et al. (2009) found species-specific bacteria in three lichens with different growth forms: fruticose, crustose, and foliose. Sierra et al. (2020) also suggested bacteria host specificity in seven genera of lichens with different thallus morphologies (*Cora*, *Hypotrachyna*, *Peltigera*, and *Sticta* are foliose, and *Usnea*, *Cladonia*, and *Stereocaulon* are fruticose). A possible explanation is that host specificity is growth-form-specific. Park et al. (2016) demonstrated that bacteria grouped together depending on the growth forms of the lichen host (crustose, foliose, or fruticose); however their sampling was rather limited. This assumption agrees with Fernández-Brime et al. (2019), who revealed that morphologically simple forms of lichenization (borderline lichens) do not influence bacterial communities, but complex thallus structure is required for the lichens to provide unique niches to host specific bacterial communities. A similar pattern was observed in green seaweeds where the bacterial variation was attributed to thallus differentiation (Morrissey et al. 2019), or in the liverwort *Marchantia inflexa* Nees and Mont., whose differences in bacteriome seem to correspond to differences in the physiology and morphology of male and female plants (Marks et al. 2017). Our study focuses only on fruticose species with similar thallus morphology, which might explain the lack of selectivity. Nevertheless, to test the hypothesis of growth form selectivity in reindeer lichens, more distantly related samples with different thallus morphologies are needed for comparison.

4.3. Geography shapes the bacterial community composition and structure

Alonso-García et al. (2021) revealed that populations of *C. stellaris* in southern Quebec were not genetically different from those of northern LWs, and suggested constant migration between populations. However, the bacterial community associated with *C. stellaris* does not follow this pattern. We found significant differences between the bacterial community of Kuujjuarapik and PNGJ's LWs. Lichens from Kuujjuarapik (northern LWs) exhibit higher diversity and abundance of bacteria. The effect of geography in the bacterial community of other lichens has been previously tested. Hodkinson et al. (2012) used different species of lichens to show that bacterial communities were significantly correlated with differences in large-scale geography (Alaska, Costa Rica, and North Carolina). At a smaller scale, the bacterial community of *L. pulmonaria* from the same sampling site showed higher similarity than those of distant populations (100 km of linear distance) (Aschenbrenner et al. 2014). Likewise, bacteria associated with *Cetraria aculeata* were clustered by geography, although contrary to our results, they were less diverse in high latitude habitats (Antarctica and Iceland) than in extrapolar habitats (Spain and Germany) (Printzen et al. 2012).

Differences in bacterial relative abundance between LWs were particularly evident for *Ca.* Eremiobacterota, a phylum usually found in acidic and cold environments (Grasby et al. 2013; Trexler et al. 2014; Bragina et al. 2015) and putatively capable of anoxygenic carbon fixation in boreal mosses (Holland-Moritz et al.

2018). The role of *Ca.* Eremiobacterota in reindeer lichens is unknown and in need of further research. Members of the family *Caulobacteraceae* (represented by species related to *Brevundimonas vesicularis*, ca. 96.6% identity) were also more abundant in northern LWs. This family has been frequently associated with lichens (Hodkinson and Lutzoni 2009; Hodkinson et al. 2012; Aschenbrenner et al. 2014; Sigurbjörnsdóttir et al. 2015; Park et al. 2016; Noh et al. 2020), but their functions have never been elucidated. Seven ASVs closely related to the anoxygenic phototrophic purple bacterium *Rhodopila globiformis* (*Acetobacteraceae*) were significantly more abundant in Kuujjuarapik. The order *Rhodospirillales* is dominant in Antarctic lichens (Park et al. 2016), and it may provide photosynthetic products, defence against pathogens and reduce oxidative stress (Cernava et al. 2017). Finally, with an identity of 94.9%, we found a close relative of *Tundrisphaera lichenicola*, significantly more abundant in northern Quebec. This species of the phylum *Planctomycetes* was described from lichen-dominated tundra soils within the zone of forested tundra and discontinuous permafrost of northwest Siberia (Ivanova et al. 2016; Kulichevskaya et al. 2017). The higher relative abundance of bacteria in northern LWs might be related to different colonization processes or to a higher presence of bacteria in northern soils. For further research, we suggest focusing on these points to understand why northern LWs harbour greater abundance of bacteria. In addition, future studies should clarify the role that *Ca.* Eremiobacterota, *Caulobacteraceae*, *Rhodospirillales*, and *Planctomycetes* play for reindeer lichens and (or) for the boreal forest to better understand patterns of diversity and abundance.

Unlike the forementioned bacteria, the genus *Endobacter* (with *Gluconacetobacter tumulioli* as the closest known relative according to the NCBI dataset) was more abundant in southern LWs. The specific role of *G. tumulioli* in the ecosystem remains unknown (Nishijima et al. 2013), although other species of this genus, such as *G. diazotrophicus*, are involved in nitrogen fixation (Saravanan et al. 2008).

4.4. Common core bacteria are reduced and homogeneous

We have defined the common core bacteria as the bacteria occurring with reindeer lichen above an occupancy frequency threshold of, at least, 0.50. Comparing 153 samples throughout eastern Canada revealed a common core of 45 ASVs representative of the families *Acetobacteraceae* and *Beijerinckiaceae* (orders *Acetobacterales* and *Rhizobiales*, respectively). All these members were also found to be associated with the single species *C. stellaris*. Sierra et al. (2020) identified a reduced (16 OTUs, threshold ≥0.90) but more diverse core in different genera of Paramos' lichens (orders *Rhodospirillales*, *Sphingomonadales*, *Rhizobiales*, *Acidobacteriales*, and the phylum *Cyanobacteria*). The core bacteria of Austrian populations of *L. pulmonaria* represented 16% of the OTUs (ca. 5% in reindeer lichens) from six phyla (*Alphaproteobacteria*, *Sphingobacteria*, *Actinobacteria*, *Nostocophycideae*, *Spartobacteria*, and *Deltaproteobacteria*), but it was considered as a regional core (Aschenbrenner et al. 2014). The reason why reindeer lichens hardly share a core bacteria might be due to the larger size of our study area. As suggested by Risely (2020), detecting the core across sites or populations can provide a reduced core bacteria. Nevertheless, we should consider that bacteria can have high or low occupancy frequency within the host population for many reasons (e.g., they are more or less common in the environment (David et al. 2014) or highly competitive against other microbes (Coyte and Rakoff-Nahoum 2019)).

BLAST hits of most of the ASVs composing the core bacteria of reindeer lichens belonged to *Methylorosula polaris* (ca. 97.93% identity), a member of the order *Rhizobiales* isolated from methane-oxidizing soil communities from the polar tundra (Berestovskaya et al. 2012). *Methylorosula polaris* had been previously detected (identity 94.5%) at the apical and middle parts of *C. squamosa* thalli (Noh et al. 2020). In general, *Rhizobiales* occur in lichens (Hodkinson and Lutzoni 2009), where they are known to perform functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation and stress protection (Erlacher et al. 2015; Cernava et al. 2017). The remaining ASVs from the core bacteria belonged to *Acetobacterales* and they might be relatives of the human pathogen *Granulibacter bethesdensis* (ca. 96.6% identity) (Greenberg et al. 2006), the phototrophic *Rhodopila globiformis* (ca. 96.4% identity), *Endobacter medicaginis* (ca. 96.1% identity) or different species of the genus *Gluconacetobacter* (ca. 95.9% of identity), involved in nitrogen-fixation (Fuentes-Ramírez et al. 2001; Saravanan et al. 2008).

Cladonia stellaris harbours almost the same common core bacteria as all reindeer lichens. The main difference is the number of ASVs, which is higher in *C. stellaris* (45 versus 87 ASVs). In addition, *C. stellaris* included *Methylocystis bryophila*, a bacterium isolated from acidic *Sphagnum* peat in Europe (Belova et al. 2013).

5. Conclusions

We provide the first assessment of the bacterial community of reindeer lichen in the boreal forest of eastern Canada. Here, we answer some key aspects of lichen bacteriome from northern ecosystems and highlight future research venues. We show a dominance of *Alphaproteobacteria* in reindeer lichens and an absence of bacteria host selectivity. Nevertheless, we also point out the need to elucidate species delimitation in reindeer lichens using phylogenomic approaches. This will improve the analyses of host selectivity of the bacteria associated with these species, and it might provide a more accurate response. In addition, our results show evidence of the influence of geography in shaping the bacterial community of reindeer lichens. A single species from one ecosystem exhibits significantly higher diversity and abundance of bacteria in northern lichen woodlands. Regarding the core bacteria, we identify a reduced core in reindeer lichens composed mainly of *Methylorosula polaris*. A deeper understanding of the interaction between reindeer lichens and *Methylorosula polaris* would help to discover ecological and functional processes at the organismal and ecosystem level.

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