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Extremely low genetic diversity of *Stigonema* associated with *Stereocaulon* in eastern Canada

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Abstract. *Stigomena* is a genus of cyanobacteria that is often the photobiont associated with the lichen genus *Stereocaulon*. To elucidate the evolutionary relationships between *Stereocaulon* and *Stigonema* and assess whether there is specificity or selectivity at the ecosystem or species levels, we performed phylogenetic analyses on specimens collected throughout Eastern Canada. We generated ITS sequences from the fungal component of the symbiosis and sequences from the operon *rbc*L-*rbc*X and the *trn*L intron from the cyanobacteria of seventy specimens of *Stereocaulon*. Our ITS results revealed that at least forty *Stereocaulon* specimens are in 16 distinct species clades (OTUs) and morphologically defined species tend to be paraphyletic. In addition, two genera of cyanobacteria, *Stigonema* and *Nostoc*, were detected among the samples and the former is the most common symbiont associated with *Stereocaulon*. We discovered that nearly invariable *Stigonema* sequences (*rbc*L-*rbc*X) occur across 2,000 km of sampling from temperate to arctic biomes. The lack of geographic structure or species-level specificity for *Stigonema* suggest a high co-dispersal capability of the cyanobacteria with the ascomycete and selectivity towards a small number of very similar *Stigonema* haplotypes across eastern Canada.

Keywords. Coevolution, nitrogen fixation, Nordic ecosystems, symbiosis, phylogenetic diversity, specificity.

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Mutualistic symbiosis provides insights on physiological, chemical and genomic interactions between partners (Spribille et al. 2016; Steinhauser et al. 2016) and is one of the most spectacular biological interactions evolutionarily and ecologically. In order to understand the role of each symbiont (e.g., tradeoffs and benefits between the partners) it is important to assess patterns of association, especially selectivity and specificity (Yahr et al. 2004). Organisms with high specificity are those with narrow requirements for symbiotic partners. An extreme example is the co-evolution of the cyanobacterium *Anabaena* with the fern *Azolla* (Li et al. 2018). The frequency of the partnership, or selectivity, describes the extent and diversity of the association and this characteristic helps to predict underlying ecological processes shaping the mutualistic interaction. Even in specific co-evolutionary systems, such as figs and wasps, it is possible to observe partner switches (Machado et al. 2005). To better understand the extent of specificity and selectivity, phylogenetics can be used, as typically done in lichens (e.g., O'Brien et al. 2005; Yahr et al. 2004)

Lichens are the symbiotic organisms or holobionts "par excellence"; they are composed of a primary fungus (typically an ascomycete) (Spribille et al. 2016), numerous bacteria (Bates et al. 2011) and a photobiont (algae and/or cyanobacteria) that

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co-exist in a composite state morphologically unlike any of its components. Lichens have historically been the subject of intense studies in almost every aspect of their symbiotic life style (Nash 2008 for a review). Additionally, the patterns of specificity and selectivity of the green algae (e.g., *Asterochloris, Chloroidium, Trebouxia*) have been addressed at fine scales (Beck et al. 2002; Yahr et al. 2004) and globally (Dal Grande et al. 2014; Singh et al. 2017; Vančurová et al. 2018).

Molecular studies on the diversity and selectivity of the cyanobacterial symbiont or cyanobionts (especially the polyphyletic *Nostoc* and *Rhizonema*) have typically used two variable markers, the *trn*L intron (Jüriado et al. 2019; O'Brien et al. 2005; Rikkinen et al. 2002) and the *rbc*L-*rbc*X operon (O'Brien et al. 2005, 2013; Rudi et al. 1998). The few global studies on lichen cyanobionts support high local diversity with ecologically specialized mycobionts associated with very few cyanobacterial phylotypes, while symbiotically generalist fungal species tend to colonize new environments and display more flexibility (Lücking et al. 2009; Magain et al. 2017; O'Brien et al. 2005, 2013; Wirtz et al. 2003).

In fact, studies on *Nostoc* in cyanolichens suggest that broadly distributed cyanobionts associated with a fungus may have a low evolutionary rate or efficient dispersal mechanisms (Magain et al. 2017; O'Brien et al. 2005, 2013; Otálora et al. 2010; Wirtz et al. 2003) or be a part of lichen guilds in bipartite lichens, in which unrelated fungal species in similar habitats share the same cyanobionts (Jüriado et al. 2019; Rikkinen et al. 2002). *Nostoc* specificity may also be explained by asexual vs. sexual dispersal mechanisms: lichen species with asexual propagules may be dispersing the cyanobacteria along with them or selecting the previously existing cyanobionts from established asexual taxa (Belinchón et al. 2015; Rikkinen et al. 2002).

In boreal eastern North America, lichens form an extensive ground layer defining the lichen woodlands (Payette & Delwaide 2018), covering up to 299,000 km^2 ($\sim$1.4 times the size of Great Britain) in the province of Quebec alone. The most common lichen woodland species include the reindeer lichens, *Cladonia* subgenus *Cladina* (e.g., *C. arbuscula* subsp. *mitis, C. rangiferina, C. stellaris*, and *C. stygia*) and the genus *Stereocaulon* (e.g., *S. condensatum, S. grande, S. paschale* and *S. tomentosum*; Brodo et al. 2001; Kershaw 1978; Payette & Delwaide 2018). *Stereocaulon* species have a mutualistic association with nitrogen-fixing bacteria within structures called cephalodia (i.e., *Nostoc* or *Stigonema,* **Fig. 2C**) (Huss-Danell 1977, 1979; Kershaw 1978; Kytövita & Crittenden 2002). Across the Canadian Shield, the potential contribution of fixed N_2 from these bacteria to nutrient-poor soils makes *Stereocaulon* an important genus in early to late successional stages of lichen woodlands (Crittenden & Kershaw 1978; Kershaw 1978).

Stereocaulon has a global distribution, with 30 species in Eastern Canada and ca. 20 species in the province of Quebec (**Fig. 1**; Esslinger et al. 2016) that need systematic revision (Vančurová et al. 2018). In eastern Canada, species of *Stereocaulon* occur throughout temperate-deciduous forests, the boreal biome, the taiga and the Arctic tundra (Brodo et al. 2001; Lamb 1951). *Stereocaulon* associates with few genera of green algae (e.g., *Asterochloris, Chloroidium*), some of which (e.g., *Asterochloris irregularis*) have a wide distribution (Vančurová et al. 2018). The typical morphology of most *Sterocaulon* species is a crustose thallus, that disappears in most species and a secondary thallus or "pseudopodetia," which support secondary ramifications called "phyllocladia." The cephalodia occur externally (unlike other lichen species such as *Lobaria pulmonaria* and *Nephroma arcticum)* on the pseudopodetia or primary thallus (e.g., brownish *Stigonema*; **Fig. 2B**). Historically, two types of cephalodia have been described in *Stereocaulon* (Johnson 1938; Lamb 1951): the small spherical type (less than 1 mm in diameter) and the sacculate type with a clear stipe and well-developed hyphal cortex. In species with the spherical type (e.g., *S. paschale* and *S. tomentosum*), a short stipe supports the colony and the hyphae are finely intertwined with the outermost external part of the cyanobacterial filaments. Also, at the base of the cephalodium, the connection between the fungus and cyanobacteria is more evident (Johnson 1938; Lamb 1951; **Fig. 2B**)

Despite considerable research on the specificity and environmental variables driving the association between *Stereocaulon* and green algae (Peksa & Škaloud 2011; Vančurová et al. 2018), there are no studies focused on the phylogenetic diversity of the cyanobionts in *Stereocaulon*, at a regional or global level (see also Lücking et al. 2009). Most of the

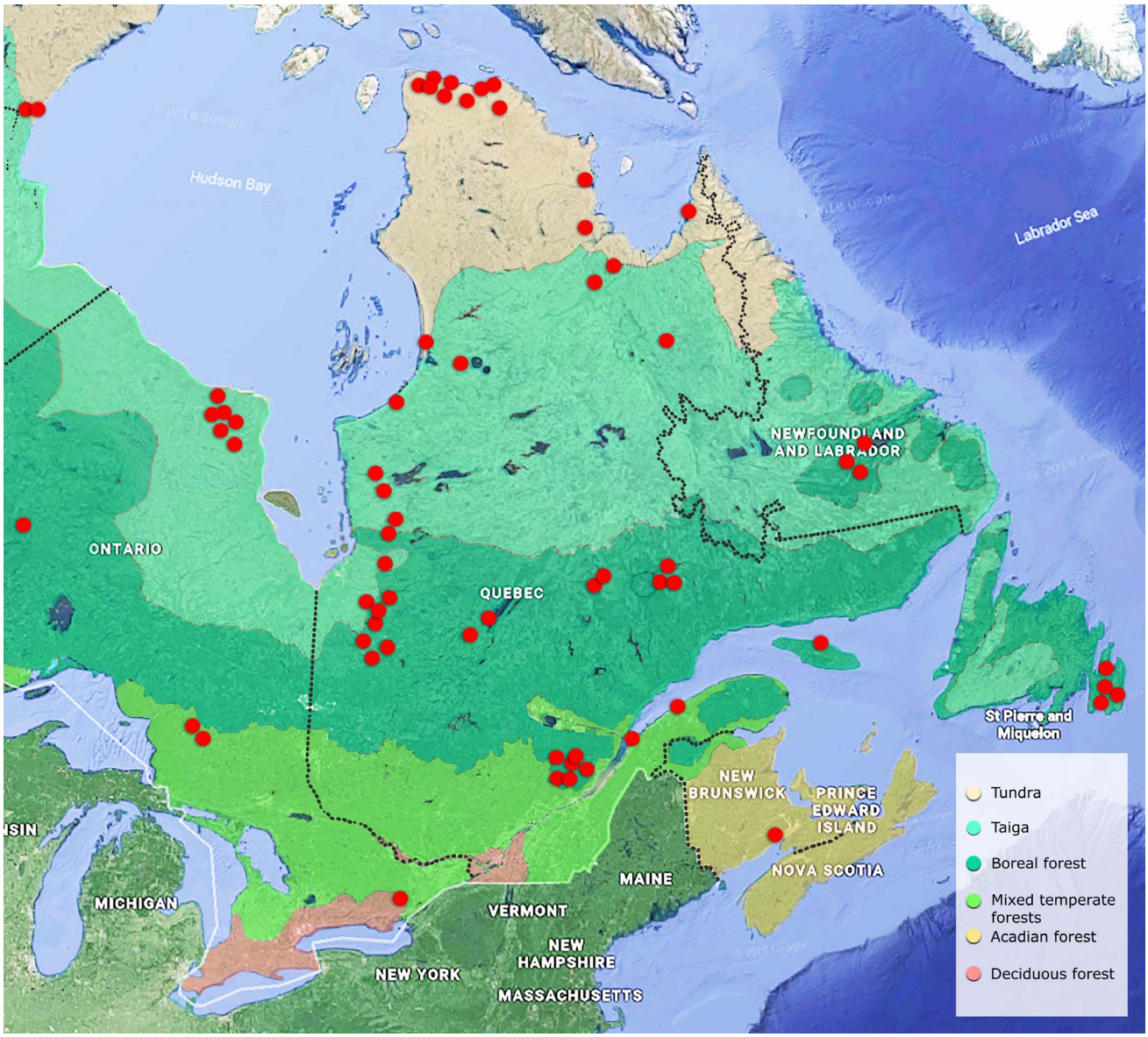

Figure 1. Map of eastern North America with collection points, see Table 1. The six different biomes included in the study are color-coded. They follow the Canadian classification from Natural Resources Canada (NRCAN). In this classification, lichen woodlands are included and coded as taiga.

information on lichen-cyanobacterial symbiosis is based on studies in the genera *Peltigera* and *Nephroma* and Collemataceae (e.g., Jüriado et al. 2019; Lücking et al. 2009; Magain et al. 2017; O'Brien et al. 2005; Otálora et al. 2010; Wirtz et al. 2003).

Here, we set out to do the first exploratory phylogenetic diversity analyses of cyanobacteria associated with *Stereocaulon*. We assess whether fungal species preferentially associate with a particular *Stigonema* or *Nostoc* phylotype (specificity) and how frequent the cyanobacterial phylotypes occur in symbiosis (selectivity). To answer these questions, we have sampled the cyanobacteria associated with *Stereocaulon* throughout eastern Canada. This region is ideal for studying latitudinal gradients because of the presence of many different biomes: the mixed coniferous-deciduous forests from Ontario and southern Quebec, represented by the bitternut-hickory-maple forests, basswood-maple forests, yellow birch-maple forests and white birch-spruce forests, the boreal zone and the lichen woodland (sometimes referred to as taiga) from middle Ontario and middle Quebec, and the least

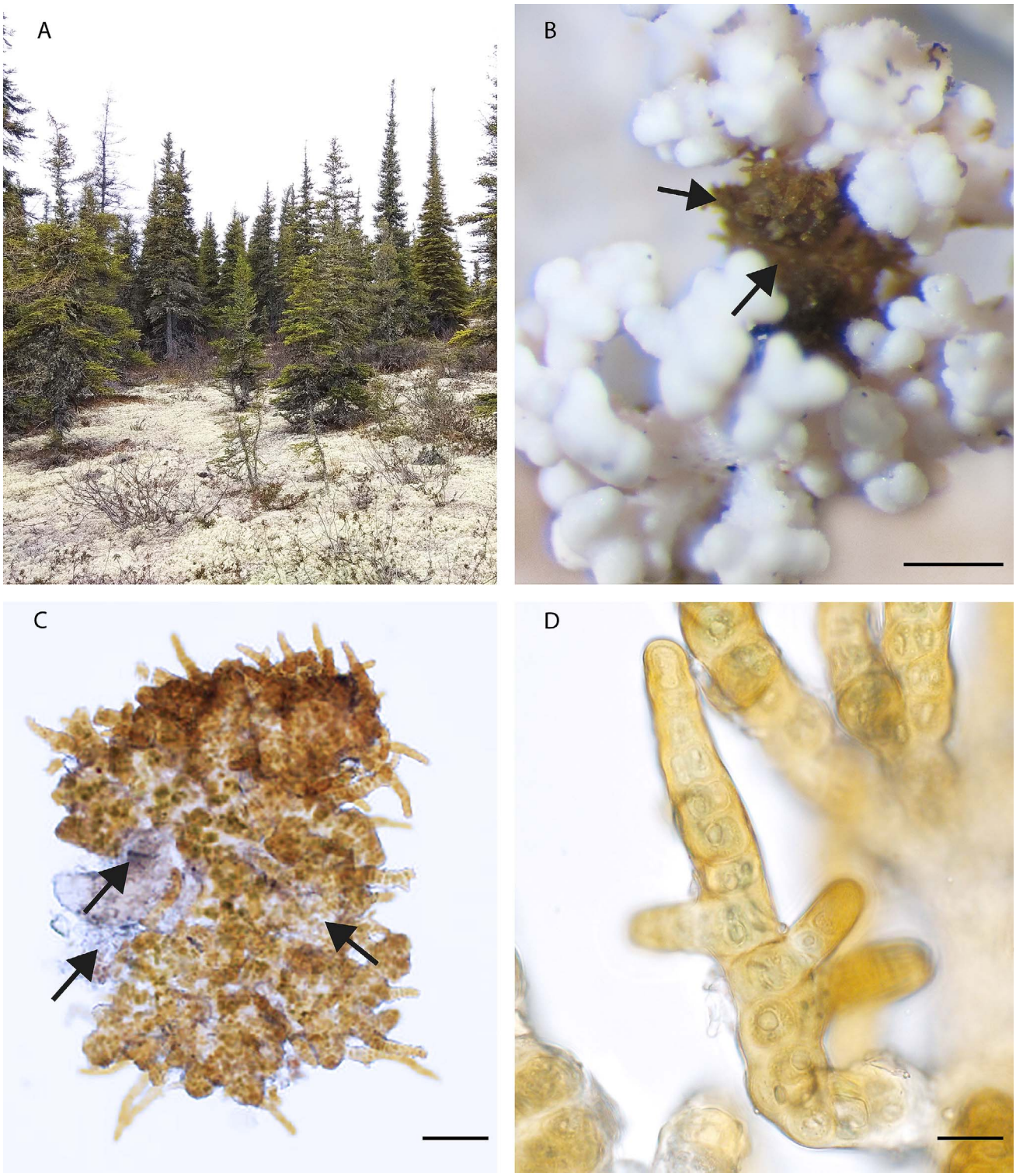

Figure 2. A. Typical habitat of *Stereocaulon* cf. *paschale* in northern Quebec, Hudson Bay. Photo by Marta Alonso García. **B.** Picture of *Stereocaulon* cf. *paschale* with the typical brown-colored cephalodia, the spheric type sensu Johnson 1938, photo by C. Lavoie. Scale= 2 mm. **C.** One cephalodium of *Stereocaulon* cf. *paschale*, showing the fungal structure around the base (arrows) of *Stigonema* cells. Scale= 1 mm. Photo by C. Lavoie. **D.** Outer filaments of *Stigonema* associated with *Stereocaulon* cf. *paschale*. Note the single or biseriate filaments. Scale= 5 mm. Photo by C. Lavoie.

accessible and logistically complex subarctic tundra (tree tundra) and shrubby-herbaceous tundra (arctic) above the 56th parallel in the Nunavik/Nunavut regions (**Fig. 1**). The aims of this study are to:

1) Infer the phylogenetic diversity and distribution of cyanobionts associated with the genus *Stereocaulon* across temperate, boreal and tundra biomes in Eastern Canada.
2) Explore patterns of association in cyanobacteria (especially *Stigonema)*, in particular clade specificity and selectivity towards a biome type and species identity.

Materials and Methods

Sampling across biomes. Seventy *Stereocaulon* specimens were collected in eastern Canada by the authors and identified using morphology and chemical spot tests following Brodo et al. (2001). Specimens represented 14 morphospecies of *Stereocaulon* with 1–23 individuals per morphospecies (**Table 1**). All samples were georeferenced (**Fig. 1**) and the sampling area was divided into six biomes or ecoregions: deciduous forests, Acadian forests, mixed temperate forests, boreal forest, taiga (including lichen woodlands) and tundra. We follow the Canadian classification for ecoregions and biomes from Natural Resources Canada (NRCAN, https://cfs.nrcan.gc.ca/assets/file/504).

Molecular markers to uncover phylogenetic diversity. To uncover the diversity of the mycobiont we used the internal transcribed spacer (ITS). The newly designed primers for the fungus are: ITS-42F (TGCGGAAGGATCATTACCAGAG) and ITS-644R(CCCTACCTGATCCGAGGTCA). To infer the phylogenetic relationships of *Stereocaulon* cyanobionts we generated nucleotide sequences from portions of the *rbc*L-*rbc*X operon and the *trn*L intron. DNA was extracted using a Plant Dneasy Kit (Qiagen). ITS and the two cyanobacterial molecular markers, *trn*L and the *rbc*L-*rbc*X operon (Rudi et al. 1998) were amplified by PCR using previously published primers. Each 25 μL PCR reaction included: 25 μg BSA, 0.626-1 U Taq DNA polymerase (Qiagen), 1.5 mM $MgCl_2$, dNTPs (0.2mM each), primers (0.5 μM forward and reverse) and 1× PCR buffer. The thermocycler temperature profile for both *rbc*L-*rbc*X and of *trn*L was: a 95°C denaturation temperature for 5 min, followed by 35 cycles at 95°C for 45 s, 52°C for 45 s and 72°C for 1:30 min, with a final extension of 72°C for 10 min. The only difference was the annealing temperature 58°C for *trn*L and 52°C for *rbc*L-*rbc*X. The primers used for PCR and sequencing are: *rbc*L-*rbc*X (cw, forward): CGTAGCTT CCGGTGGTATCCACGT; *rbc*L (cx, reverse): GGGGCAGGTAAGAAAGGGTTTCGTA. For *trn*L (Leu1, forward): TGTGGCGGAATGGTAGACGC TAC and *trn*L (Leu2, reverse): GACTTGAACCCA CACGAC.

We generated 40 ITS sequences, 55 *rbc*L-*rbc*X sequences, and 67 *trn*L sequences. Voucher information and GenBank accession numbers are provided in **Table 1**.

Phylogenetic analyses; species delimitation analyses of the mycobiont. We assessed the taxonomic status of the populations of *Stereocaulon* included in the phylogenetic study and used those available from GenBank. We applied three species delimitation methods, the Poisson Tree Process using a maximum likelihood framework (MLPTP), the Poisson Tree Process using a Bayesian framework (BPTP) and multi-rate Poisson Tree Process (mPTP) (Kapli et al. 2017). We analyzed the data with the General Mixed Yule Coalescent (GMYC) using a single and multiple threshold and we recovered completely different results. A single threshold is usually recommended based on simulations (not from empiric data) and tends to over split species (Correa et al. 2017; Zhang et al. 2013). Our preliminary runs resulted in three species clusters using a single threshold. In contrast, multiple thresholds resulted in sixty-two species clusters. We opted for using three PTP methods because we could not explain such an incongruence and because the GMYC analyses require a calibrated tree. A single locus analysis of ITS does not have the resolution for every species cluster and the program BEAST is known to create dichotomies (as non-zero branch lengths) biasing the outcome of the analysis.

PTP methods model interspecies processes by using a two-parameter model, a parameter for speciation and another for coalescent processes and provides support either using a ML approach (MLPTP) or a Bayesian approach (BPTP) based on number of expected substitutions. A newly developed algorithm based on PTP (mPTP), aimed at improving estimates for species with distinct rates of speciation and coalescence. The mPTP allows for

different rates of speciation events within each inferred species. We downloaded *Stereocaulon* sequences from GenBank (359 sequences), mostly from Vančurová et al. (2018) and Högnabba (2006). We then trimmed identical sequences from the database using Geneious. The final dataset contained 247 taxa and included two *Lepraria* species as an outgroup (**Supplementary Table S1 & Supplementary Alignment S1**). We analyzed the ITS locus using a maximum likelihood criterion (ML) and the GTRCAT model implemented in RAxML (Stamatakis 2014) with 500 bootstrap replicates. The MLPTP and BPTP analysis were performed on a maximum likelihood tree from RAxML on the online server (http://species.h-its.org/ptp/). The analysis was run for 500,000 generations, using 0.4 burn-in and 100 thinning. In contrast, the mPTP analysis was done using default settings and using the web server (https://mptp.h-its.org/).

Phylogenetic diversity of the cyanobionts. To investigate the phylogenetic diversity of the cyanobionts using the two markers we used three datasets. The first dataset comprises 589 taxa from Magain et al. (2017) and O'Brien et al. (2003) that included cyanobacteria associated with lichens, plants (hornworts, *Blasia*, cycads, *Gunnera*), lichens (e.g. *Rhizonema*) and free-living strains such as *Nodularia* and *Fischerella*. Additionally, we added GenBank sequences of two free-living species of *Stigonema*, *S. dinghuense* (KJ786938) and *Stigonema* sp. (KT868763). Two important modifications were done to the alignment: first, we excluded the *rbc*L-*rbc*X spacer due to known alignability issues (Magain et al. 2017) and eliminated identical sequences resulting in 536 accessions (**Supplementary Table S2 & Supplementary Alignment S2**). The second matrix (**Supplementary Alignment S4**) consisted of a more reduced dataset (64 sequences) with the sequences generated in the study and two free-living species of *Stigonema*, *S. dinghuense* (KJ786938) and *Stigonema* sp. (KT868763) (**Fig. 5**).

To analyze the *trn*L intron, we used a dataset of 102 sequences (including ours) and those from O'Brien et al. (2005) (**Supplementary Table S3 & Supplementary Alignment S3**). Even though there are over a thousand *trn*L sequences on GenBank, we used a reduced dataset for the following reasons: the *trn*L locus is very small and less informative than the *rbc*L-*rbc*X region (**Supplementary Tables S2 & S4**).

We analyzed each locus and dataset separately under the maximum likelihood criterion (ML) using the GTRCAT model approximation implemented in RAxML (Stamatakis 2014) with 500 bootstrap replicates. Basic statistics on each dataset were retrieved using PAUP* (Swofford 2002). Bayesian analyses of each marker were conducted in MrBayes 3.2 (Ronquist et al. 2012), using the default two runs and four chains, with default priors on most parameters and a model chosen by mrModeltest (Nylander 2004). To assess burn-in and convergence we compared the bipartitions across the two runs and a visual assessment using Tracer. Convergence was usually achieved after 2 million generations, with trees sampled every 10,000th generation for a total length of 20,000,000 generations; we discarded 25% of each run and then pooled the runs. All analyses were run under the CIPRES platform (Miller et al. 2010).

Haplotype network. Forty-two accessions of eastern North America *Stigonema* were used to build a haplotype network using PopArt (Clement et al. 2002; Leigh & Bryant 2015). We analyzed the dataset by ecological region (or biome) for both *rbc*L-X and *trn*L, but we only present *rbc*L-X.

Results

Species delimitation analyses of the mycobiont. Forty sequences were added to existing published sequences of *Stereocaulon*. The MLPPT recovered 55 species, the BPPT recovered 96 species and the mPTP recovered 35 species (**Fig. 5**, **Supplementary Fig. S1**). Most of the supported species using the BPTP (posterior probability of 1.0) and MLPTP (support of 100%) coincide with the clusters provided by the mPTP analysis (**Supplementary Fig. S1**).

The phylogenetic analysis of the ITS sequences of *Stereocaulon* mycobionts shows that many of the morphospecies are paraphyletic, if we follow the species definition proposed by Högnabba (2006). Our samples fall in 16 different OTUs. Most sequences uploaded by Vančurová et al. (2018) lacked species names and our samples do not seem to belong to any of the species proposed by Högnabba (2006), except *S. vesuvianum* and *S. virgatum*. A manual mapping of the species against the reduced *rbc*L-X tree shows that some species cluster that come from distant regions like Labrador

Table 1. Cyanobacterial (*rbc*L-X, *trn*L) species associated with the genus *Stereocaulon* and data bank accession numbers. Numbers in parenthesis are collection points located on the map (Fig. 1).

Fungal morphospecies host species	Sequenced cyanobiont genus	Biome or ecoregion		GenBank accession no.				
				Cyanobacteria		Fungus	Voucher accession no.	Collector
				*rbc*LX	*trn*L	ITS		
Stereocaulon sp.	*Nostoc*	Tundra	Kuujjuarapik, Quebec, Canada	—	MK040927	MN596949	CA-16-1550	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Tundra	Tasiujaq, Nunavik, Canada	MT273227	MK040923	—	SF13-1218	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Tundra	Hudson Strait, Nunavik, Canada	—	MK040925	—	SF15-1121	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Tundra	Polar Bear Provincial Park, Ontario, Canada	—	MK040926	MN596950	CANL 124952	Binker, S.R.
Stereocaulon sp.	*Nostoc*	Tundra	Arviat, Nunavut, Canada	MT273243	MK040928	—	18053	McMullin, R.T.
Stereocaulon sp.	*Nostoc*	Tundra	Arviat (West), Nunavut, Canada	MT273242	MK040930	—	18055	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Taiga	Kenora County, Ontario, Canada	MT273237	MN596290	MN596950	CANL124952	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Tundra	Hudson Strait, Nunavik, Canada	MT241308	MK040932	MN596952	SF15-1316	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Tundra	Hudson Strait, Nunavik, Canada	—	MN596291	—	SF15-1229	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Tundra	Hudson Strait, Nunavik, Canada	—	MN596292	MN596953	SF15-1206	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Boreal	Uapishka, Quebec, Canada	MT273224	MK040933	MN596954	SF15-1782	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Tundra	Salluit, Nunavik, Canada	MT273249	MK040929	MN596955	SF15-1052	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Taiga	Koksoak-River, Nunavik, Canada	MT273230	MK040934	MN596956	SF13-0468	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Boreal	Blanchet Lake, Quebec, Canada	—	MK040935	—	QFA572618	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Boreal	Mouchalagane-River, Quebec, Canada	MT273223	MK040936	—	SF15-1779	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Boreal	Happy Valley, Labrador, Canada	MT273209	MK040937	—	17168	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Boreal	Matagami Lake, Baie James, Quebec, Canada	MT273204	MK040939	MN596957	CA-17-1740	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Acadian	Fundy National Park, Canada	MT273233	MK040976	—	QFA600843	Roy, C.
Stereocaulon sp.	*Stigonema*	Boreal	Salmonier Line, Newfoundland, Canada	MT273212	MK040940	MN596958	16063	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Boreal	Gaulin Lake, Baie James, Quebec	MT273232	MK040941	—	QFA572629	Lamarre, J.-F.
Stereocaulon sp.	*Nostoc*	Boreal	Tarp Lake, Balmertown, Ontario, Canada	MT273246	MK040931	—	CANL 124928	Binker, S.R.
Stereocaulon sp.	*Nostoc*	Boreal	Montmorency Forest, Quebec, Canada	MT273245	MK040924	MN596959	PA-CAN-030	Picord, P.
Stereocaulon sp.	*Nostoc*	Taiga	Wemindji Road, Baie James, Quebec, Canada	MT273239	MK040942	MN596960	CA-17-1731B	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Taiga	Baie James Road, Quebec, Canada	MT273206	MK040967	—	CA-17-1737	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Matagami Lake, Baie James, Quebec, Canada	MT273203	MK040943	—	CA-17-1741	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Matagami Lake, Baie James, Quebec, Canada	MT273202	MK040944	—	CA-17-1743	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Radisson, Quebec, Canada	—	MN596293	MN596961	CA-16-1545	Villarreal, J.C.
Stereocaulon sp.	*Nostoc*	Tundra	Kangirsuk, Quebec, Canada	—	MN596294	MN596962	SF15-1728	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Taiga	Baie James Road, Quebec, Canada	MT273240	MK040946	MN596963	CA-17-1678	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Camps Cree, Baie James, Quebec, Canada	MT273205	MK040945	MN596964	CA-17-1739	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Taiga	Kuujjuarapik, Quebec, Canada	MT241307	MK040947	MN596966	CA-16-1551	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Mont Valin, Quebec, Canada	MT273234	MK040948	—	QFA562782	Savard, J.
Stereocaulon sp.	*Stigonema*	Taiga	Clearwater River, Hudson Bay, Quebec, Canada	MT273231	MK040949	—	QFA541737	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Mixed temperate	Bic National Parc, Quebec, Canada	MT273229	MK040950	—	QFA614964	Anderson, F.
Stereocaulon sp.	*Stigonema*	Boreal	Mont Valin, Quebec, Canada	MT273228	MK040951	—	QFA562781	Savard, J.
Stereocaulon sp.	*Stigonema*	Taiga	Droissy Lake, Koksoak-River, Nunavik, Canada	MT273226	MK040952	—	SF12-4106	Gagnon, J.

Table 1. Continued.

Fungal morphospecies host species	Sequenced cyanobiont genus	Biome or ecoregion		GenBank accession no.				
				Cyanobacteria		Fungus	Voucher accession no.	Collector
				*rbc*LX	*trn*L	ITS		
Stereocaulon sp.	*Stigonema*	Boreal	La Porte-des-Bouleaux, Monts Groulx, Quebec, Canada	MT273225	MK040953	—	SF15-1781	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Boreal	Matagami, Quebec, Canada	MT273222	MK040955	MN596983	CA-17-1677A	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Montmorency Forest, Quebec, Canada	MT273221	MK040957	MN596967	CA-17-1676	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Boreal	Saint-Urbain, Quebec, Canada	MT273217	MK040958	MN596968	17-11.1	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Boreal	Saint-Urbain, Quebec, Canada	MT273244	—		17-11.1	Gagnon, J.
Stereocaulon sp.	*Stigonem*	Boreal	Happy Valley, Labrador, Canada	MT273215	MK040960	MN596969	17157	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Boreal	Happy Valley, Labrador, Canada	MT273214	MK040961	MN596970	17161	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Boreal	Salmonier Line, Newfoundland, Canada	MT273213	MK040962	MN596971	16078	McMullin, R.T.
Stereocaulon sp.	*Stigonema*	Taiga	Duxbury Lake, Baie James, Quebec, Canada	MT273208	MK040963	—	CA-17-1733	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Taiga	Nord Gas, Baie James, Quebec, Canada	MT273207	MK040964	—	CA-17-1735	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Taiga	Cochrane (North), Ontario, Canada	—	MN596296	MN596972	CANL127880	Lewis, C.
Stereocaulon sp.	*Stigonema*	Taiga	Kenora County, Ontario, Canada	MT273200	MN596298	MN596973	CANL124947	Oldham, M. J.
Stereocaulon sp.	*Stigonema*	Taiga	Kenora County, Ontario, Canada	MT273201	MN596297	MN596965	CANL124901	Oldham, M. J.
Stereocaulon sp.	*Stigonema*	Taiga	Kenora County, Ontario, Canada	MT273199	MN596299	MN596974	CANL124903	Binker, S.R.
Stereocaulon sp.	*Stigonema*	Taiga	Kenora County, Ontario, Canada	MT273198	MN596300	MN596975	CANL124899	Oldham, M. J.
Stereocaulon sp.	*Stigonema*	Boreal	Anticosti, Quebec, Canada	—	MN596301	—	QFA504248	Roy, C.
Stereocaulon sp.	*Nostoc*	Tundra	Hudson Strait, Nunavik, Canada	—	MN596302	MN596976	SF15-0359	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Deciduous forests	Crow Lake, Ontario, Canada	MT273220	—	—	CANL 125962	Freebury, Colin E.
Stereocaulon sp.	*Stigonema*	Boreal	Montmorency Forest, Quebec, Canada	MT273219	MK040966	MN596977	PA-CAN-029	Picord, P.
Stereocaulon sp.	*Stigonema*	Tundra	Kangirsuk, Quebec, Canada	—	MN596303	MN596978	63.7b	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Tundra	Ivujivik, Nunavik, Canada	MT241309	MK040968	MN596979	SF15-0166	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Tundra	Ivujivik, Nunavik, Canada	MT273236	MK040977	MN596980	SF15-1531	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Taiga	Kuujjuaq, Koksoak-River, Quebec, Canada	—	MK040969	MN596981	SF13-0644	Gagnon, J.
Stereocaulon sp.	*Nostoc*	Mixed temperate	Ile-Verte, Quebec, Canada	—	MK040970	—	QFA576933	Roy, C.
Stereocaulon sp.	*Nostoc*	Tundra	Kangirsuk, Quebec, Canada	MT273247	MK040971	MN596982	SF15-0641	Gagnon, J.
Stereocaulon sp.	*Stigonema*	Mixed temperate	Wakami Lake, Ontario, Canada	MT273218	—	MN596984	CANL125234	Brodo, I. M.
Stereocaulon sp.	*Stigonema*	Boreal	Matagami, Quebec, Canada	MT273216	MN596304	MN596985	CA-17-1677B	Villarreal, J.C.
Stereocaulon sp.	*Nostoc*	Boreal	Duxbury Lake, James Bay, Quebec, Canada	MT273238	MK040972	—	CA-17-1734	Villarreal, J.C.
Stereocaulon sp.	*Stigonema*	Mixed temperate	Wakami Provincial Park, Ontario, Canada	—	MN596305	—	CANL125234	Brodo, I. M.
S. vesuvianum Per. (1)	*Stigonema*	Tundra	Katinniq, Nunavik, Canada	MT273235	MK040973	—	QFA 594741	Gagnon, J.
S. vesuvianum (2)	*Nostoc*	Tundra	Sanirasiq Lake, Koksaok-River, Quebec, Canada	MT273248	—	—	SF13-0332	Gagnon, J.
S. vesuvianum (3)	*Stigonema*	Boreal	Salmon Waters Lake, Newfoundland, Canada	MT273210	MK040978	MN596987	16148	McMullin, R.T.
S. vesuvianum (4)	*Stigonema*	Boreal	Salmonier Line, Newfoundland, Canada	MT273211	MK040974	MN596986	16157	McMullin, R.T.
S. virgatum Ach.	*Nostoc*	NA	StKitts, West Indies	MT273241	MK040975	MN596988	15289	McMullin, R.T.

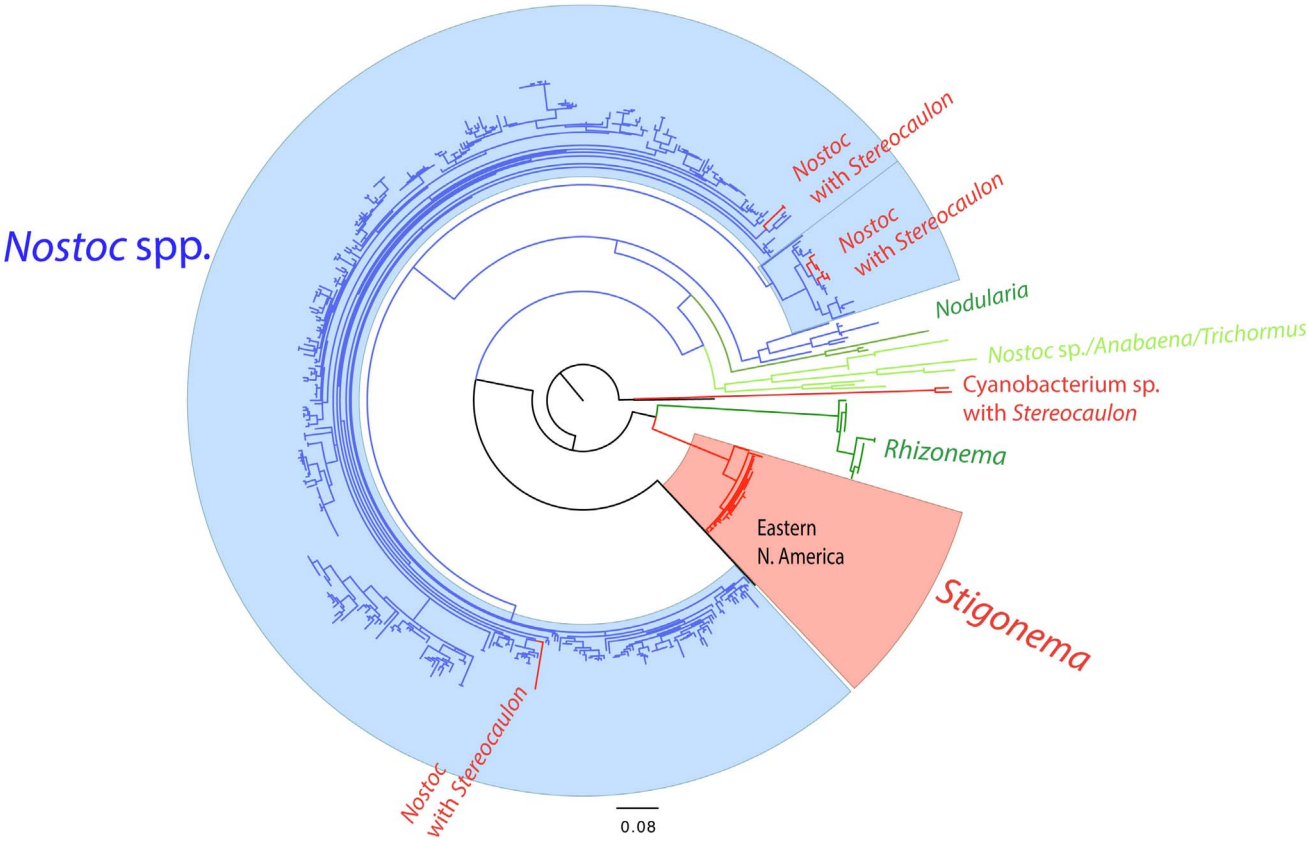

Figure 3. Maximum likelihood tree of the *rbc*L-*rbc*X operon using 536 taxa. The phylogeny shows the sister position of *Stigonema* and *Rhizonema* and the very short branches of *Stigonema* accessions. The phylogeny is overrepresented by species of *Nostoc* associated with lichens and representatives of plant cyanobionts. The few *Nostoc* accessions associated with *Stereocaulon* appear dispersed throughout the tree, including a few sequences completely isolated from any of the sampled genera.

(JC987) and Ontario (JC2017) share the same cyanobiont (**Fig. 5**).

Phylogenetic diversity using rbcL-rbcX and trnL. We generated sequences from 70 specimens across temperate, boreal, subarctic and arctic biomes (**Table 1**, **Supplementary Table S2**; **Figs. 3–5**). The length and number of informative sites for each of the three markers used for the cyanobacteria phylogeny are reported in **Supplementary Table S4**. Bayesian and ML analyses of the cyanobacteria data matrix resulted in a well-supported phylogeny, with major ingroup nodes receiving posterior probability values >0.95 and bootstrap values >79–90% (**Figs. 3–5**), except within *Stigonema*.

The global *rbc*L-*rbc*X phylogeny (536 taxa) shows an overrepresentation of *Nostoc* sequences in symbiosis with lichens (and plants) and two groups of *Nostoc* sequences associated with *Stereocaulon*. One sequence (JC997) is nested within cyanobacteria associated with *Peltigera hymenina* (KX922897, Newfoundland, Canada) and the other accessions (JC911, JC991, JC860, JC1000, JC907, JC986, JC998, JC980) are clustered near cyanobacteria associated to *Peltigera polydactylon* (e.g., KX922949) from Quebec, Norway and Japan. Two accessions (JC 981 and JC996 associated with *S. virgatum*) are found in a long branch associated to neither of the genera sampled. The *Stigonema* samples form a monophyletic group sister to *Rhizonema*. The *Stigonema* clade has the shortest branches in the phylogeny, especially the symbiotic taxa included in our study (**Figs. 3 & 5**). The reduced phylogeny gathers three groups and two well-supported clades: the *Stigonema* clade and the *Nostoc* clade (**Figs. 3 & 5**) with three sequences not nested in either of the clades (*Stereocaulon* cf. *virgatum* JC996, *Stereocaulon sp*. JC981). The *Nostoc* clade includes three species clusters of *Stereocaulon* (including *S. vesuvianum*). The two free-living *Stigonema* are sister to all the symbiotic accessions.

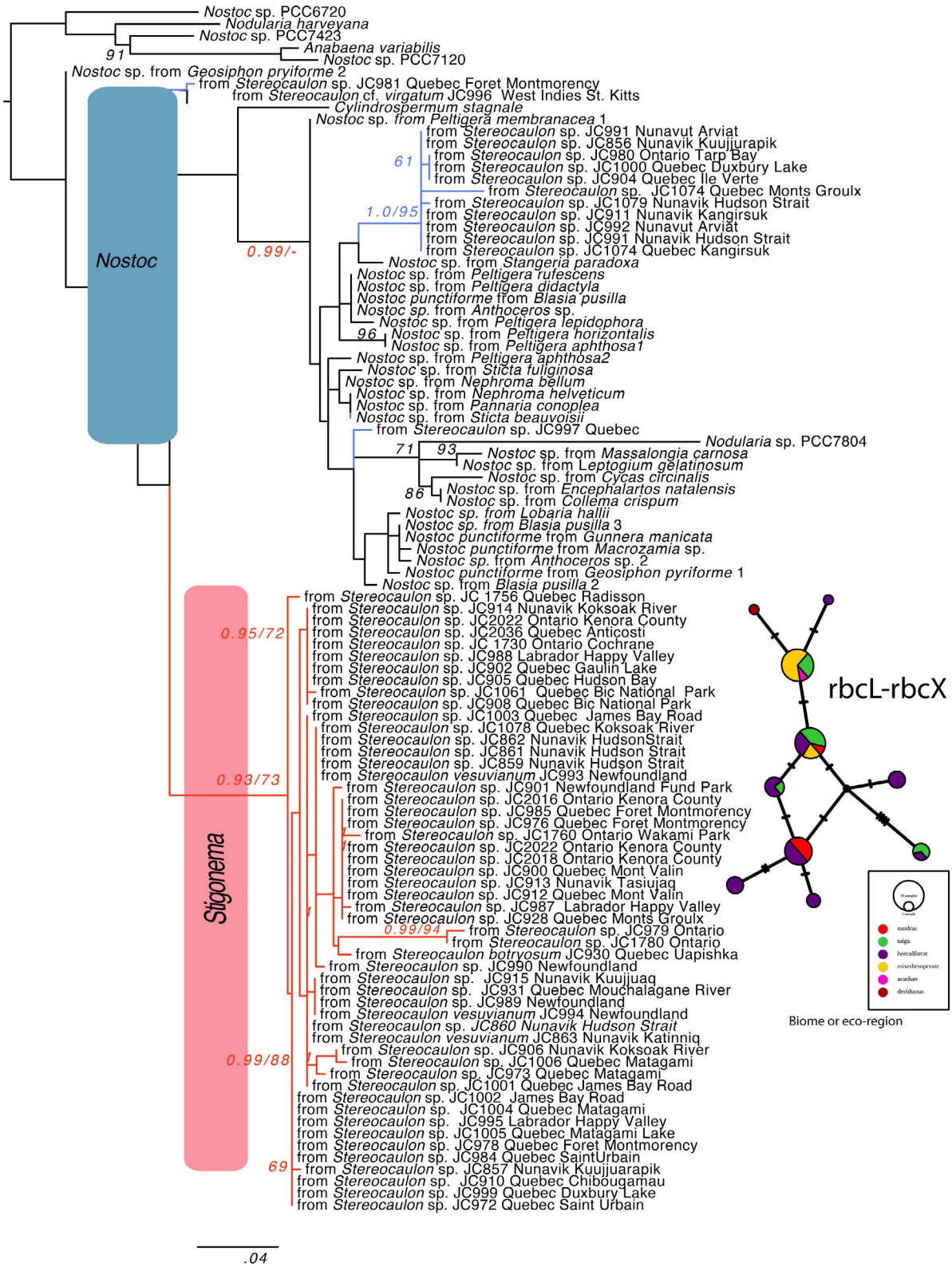

Figure 4. Maximum likelihood tree of the *trn*L intron, the tree displays the posterior probabilities from each clade indicated on top of the branches and ML bootstrap values. No evident geographical or ecosystem pattern can be inferred from the phylogeny. A *rbc*L-X haplotype network of only symbiotic *Stigonema* samples shows no specific grouping by biome.

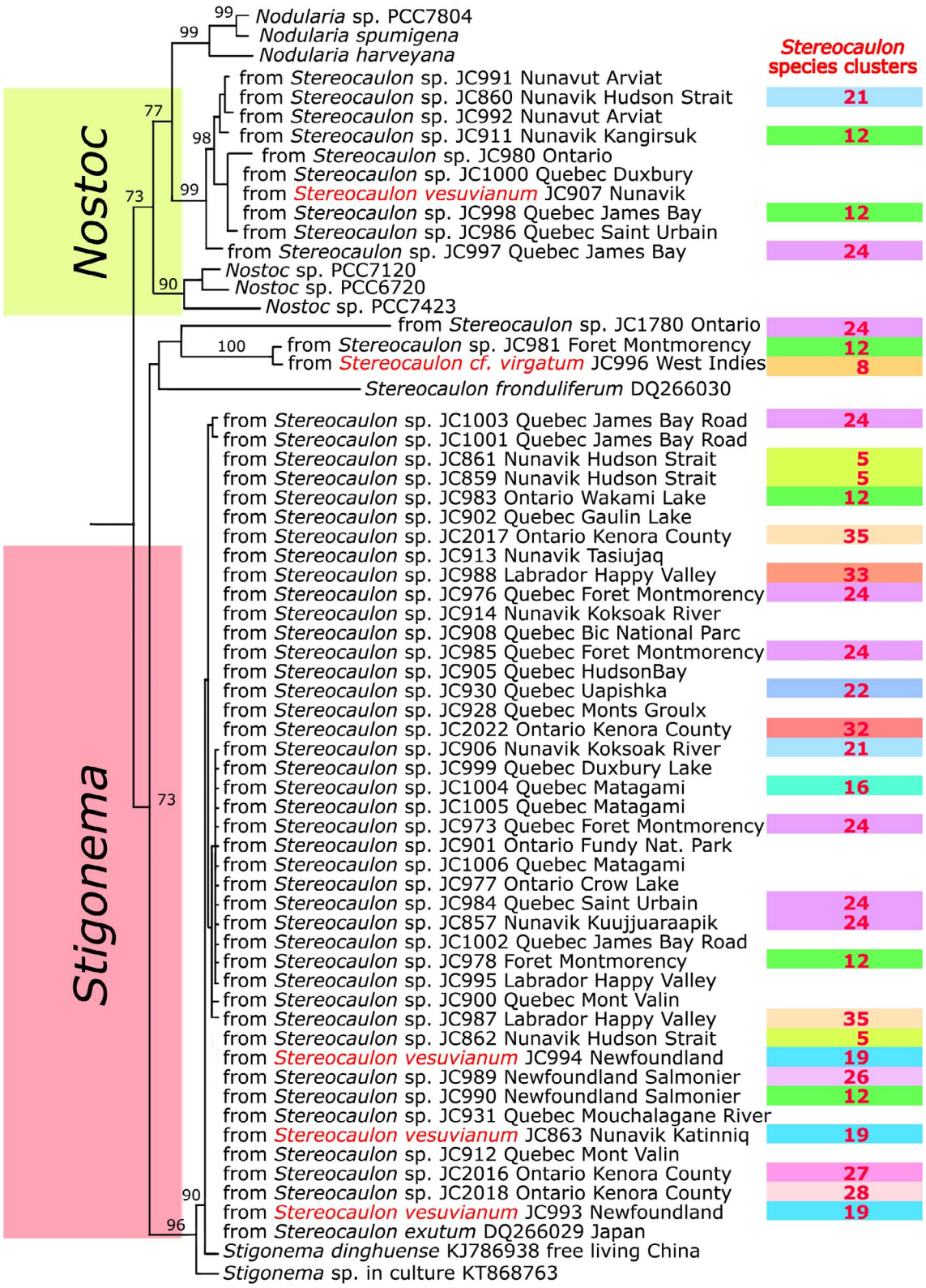

Figure 5. Maximum likelihood tree of the *rbc*L-*rbc*X operon including our sequences and three *Stigonema* sequences from GenBank. The tree displays the ML bootstrap values. The phylogeny shows the low diversity (short branches) in *Stigonema*. On the right, fungal species delimitation results from ITS using the three methods (MLPTP, mPTP and bPTP) are presented, see Supplementary Fig. S1 for full results. We discuss in the text the most conservative approach (mPTP) and numbered all the species clusters from bottom to top (1–35). To the right of the cyanobacterial accession, we have included the number of the species cluster.

The *Stigonema* clade is poorly resolved with at least two clades with short branches (**Fig. 5**).

The *rbc*L-*rbc*X spacer was excluded from the phylogenetic analyses because of problems aligning the region across *Nostoc* and *Stigonema*. However, the *Stigonema* haplotypes were identical even in this extremely variable spacer region (unalignable across the *Nostoc* strains included in this study). An analysis of the spacer among samples of the *Stigonema* clade revealed only nine SNPs, with a frequency of at least 25%. One 7bp-indel is shared by accessions from the Japanese *S. exutum* and *S. vesuvianum* from Newfoundland (JC993). The *rbc*L-*rbc*X haplotype network by biome (or ecoregions) shows the most abundant haplotypes shared by 2–4 biomes. The nucleotide diversity is p=0.049 with 11 segregating sites and 4 informative sites. The most abundant haplotypes occur in all biome categories.

The *trn*L tree is less resolved in the backbone and shows the same general topology of *rbc*L-*rbc*X, but with little support for *Stigonema* (**Fig. 4**). A single accession of *Nostoc* associated with *Stereocaulon sp*. (JC997) is outside of the *Nostoc* clade, as well as the cyanobacteria from *S.* cf. *virgatum* and *Stereocaulon* sp. (JC981). The *trn*L haplotype network shows sixteen *Stigonema* haplotypes with 10 private haplotypes (results not shown).

Discussion

Complex symbiotic relationships between the mycobiont and the cyanobiont of *Stereocaulon* species have profound implications for the survival, functional ecology and evolution of the partners. We have completed the first survey of the diversity of cyanobacteria associated with this genus, which is an important component of lichen woodlands in northern ecosystems.

Our study provides evidence of low phylogenetic diversity (very short branch lengths) in the *Stigonema* photobiont associated with *Stereocaulon* across different species clusters and biomes. For example, some species such as *Stereocaulon* from the Hudson Strait (arctic tundra) and from Newfoundland (boreal forest) have an identical cyanobiont despite being 2,000 km apart. We provide evidence of low genetic diversity throughout a large area (eastern Canada) and across different species, suggesting fidelity to a single strain (with few haplotypes) of *Stigonema* associated with the genus *Stereocaulon*. Herein, we discuss the apparent genetic uniformity of *Stigonema* across different biomes and its ecological and evolutionary implications.

Species identification and delimitation. Mycobiont species delimitation is crucial to infer selectivity and specificity in symbiotic systems. The genus *Stereocaulon* is taxonomically complex (Lamb 1951; Vančurová et al. 2018). Therefore, only specimens that could be reliably identified were included in our study, which resulted in 11 morphospecies (*S. alpinum*, *S. arenarium, S. botryosum*, *S. condensatum*, *S. dactylophyllum*, *S. grande*, *S. saxatile*, *S. symphycheilum*, *S. tomentosum* and *S. vesuvianum*). We generated ITS sequences the fungal component and evaluated the morphospecies concepts. Species delimitation analyses are not an infallible approach to discriminate between taxa (Dellicour & Flot 2018) and our results should be considered preliminary. Our fungal phylogeny and analyses recovered at least 16 distinct species lineages. However, except *S. vesuvianum*, none of the previously identified morphospecies represented monophyletic entities. Our results do not show any pattern of selectivity for the cyanobionts, nor at the phylospecies level or the morphospecies level.

A recent study also highlights the need for a systematic re-assessment of *Stereocaulon* at a global scale (Vančurová et al. 2018). The high morphological variability within current species concepts and the convergence of many key traits used to separate species (e.g. cephalodia presence/absence; pseudopodetia morphology) pose difficulties. Additionally, only two samples from eastern or central Canada were included in all phylogenetic studies available to date. The next step could be the use of multilocus approaches and a taxonomic revision to assess the position of the phylotypes in our phylogenies (see Chagnon et al. 2019; Pardo-De la Hoz et al. 2018).

Evolution and ecology of symbiotic specificity and selectivity. The relationship we recovered between the cyanobacterial diversity associated with *Stereocaulon* mostly concurs with Brodo et al. (2001), which indicate that *Stigonema* is the primary symbiont of *Sterecaulon*, and *Nostoc* only occurs in a smaller number of species or as a secondary cyanobiont. Our phylogenies, based on *rbc*L-*rbc*X and *trn*L show at least three clades: a *Stigonema* clade with little to no diversification, a more structured *Nostoc* clade, and an isolated clade,

composed of unidentified cyanobionts from three different *Stereocaulon* species clusters (*S.* cf. *virgatum*, JC996, *Stereocaulon* sp., JC981, *Stereocaulon* sp., JC1780) (**Figs. 1–5**).

***Associations with* Stigonema.** The predominance of *Stigonema* in both phylogenies was expected as their abundance among *Stereocaulon* species was historically a key feature to for identifications (Brodo et al. 2001; Johnson 1938; Lamb 1951). However, this low genetic diversity over a large territory is unexpected and not comparable with the genetic diversity in *Nostoc* (**Fig. 3**; Magain et al. 2017). Some species such as *Stereocaulon* sp. from the Hudson Strait (arctic tundra) and from Newfoundland (boreal forest) have an identical cyanobiont despite being 2,000 km apart. Moreover, there are reasons to suspect this pattern could extend further as some *Stigonema* strains (acc. KJ786938) and *Stereocaulon* species also found in tropical regions (Büdel 1999; Vančurová et al. 2018).

Stereocaulon seems to have specificity and selectivity for *Stigonema* at the genus level, as an almost unique cyanobacterial haplotype is shared by several fungal phylotypes (**Fig. 5**). To further understand what drives this singular symbiotic pattern, many aspects of the ecology of both *Stigonema* and *Stereocaulon* will need to be explored in greater detail. Nevertheless, here we present some of the plausible hypotheses.

A first explanation for such a low genotypic diversity across species and biomes is a "harvesting scenario." Under this scenario, the ancestral *Stereocaulon* species had a successful encounter with one lineage of *Stigonema*. After that, the fungus successfully diversified and dispersed in different areas with its optimal cyanobionts. Diverse stochastic environmental constraints may have triggered diversification of the ascomycete and slowly optimized the cyanobionts to perform the nitrogen-fixation tasks without the need to further sample other *Stigonema*. This hypothesis could explain how the mycobiont and the cyanobacteria, thought to co-disperse and evolve together, seem to have a different speciation speed. Thus, successful asexual reproduction, through fragmentation dispersal, could be responsible for the stability of the symbiosis (as proposed by Magain et al. 2018; Pardo-De la Hoz et al. 2018).

Another potential explanation is the low diversity of symbiotic capable *Stigonema* in eastern Canada due to the harsh and prolonged winter conditions, mirroring the situation in Antarctica (Wirtz et al. 2003). To further test this hypothesis in our system, free-living *Stigonema* around *Stereocaulon* should also be sampled. For example, the morphologically similar species, *Stigonema minutum*, could be targeted as it is common in biological soil crusts from subpolar regions (Patova et al. 2016) and is also associated with several Arctic and Antarctic moss species (Ohtani & Kanda 1987; Pandey et al. 1992; Stewart et al. 2011).

Finally, it is also plausible that the markers used for our study do not have the appropriate substitution rate to address population or species level differences in *Stigonema*. However, the *rbc*L-*rbc*X operon is routinely used to address biogeographic patterns of selectivity and specificity (Fernández-Martínez et al. 2013; Magain et al. 2017; O'Brien et al. 2005, 2013) and previous analyses show their utility for other genera such as *Scytonema*, *Rhizonema* and *Nodularia* (Lücking et al. 2009; O'Brien et al. 2005, 2013).

Association with* Nostoc *and other strains. One fifth of samples that we successful generated sequences for were associated with *Nostoc*. These *Nostoc* strains, contrary to *Stigonema,* are diversified and unspecific to *Stereocaulon* when included in a broader phylogeny. Moreover, some *Stereocaulon* phylospecies (#12, 21 & 24) are also found in association with both cyanobacterial genera at the same time. In this case, *Nostoc* may be the secondary symbiont. While extracting DNA, we took care to collect only the cephalodia in order to identify the main cyanobiont. However, further microscopic observations revealed the occasional presence of *Nostoc* filaments in the proximity of the cephalodia.

Previous work on *Collema*, *Leptogium*, *Lobaria*, *Nephroma*, *Parmeliella*, and *Peltigera* already concurred with the fact that lichens seem to share preferential symbiotic *Nostoc* strains; even though the patterns of specificity at the species level vary considerably among studies (Chagnon et al. 2019; Lohtander et al. 2003; Magain et al. 2017, 2018; Pardo-De la Hoz et al. 2018; Paulsrud et al. 2000; Rikkinen et al. 2002; Stenroos et al. 2006; Wirtz et al. 2003). Our results show that *Stereocaulon* may benefit from the presence of other lichens and share local symbionts. It is possible, as it was suggested for *Peltigera* by Magain et al. (2018), that events following sexual reproduction in favorable areas

lead to partner loss (*Stigonema*) and thus, the use of *Nostoc* strains (or else) from the immediate environment; likely from the soil, other lichens, or mosses.

Conclusion

Complex symbiotic relationships between the mycobiont and the cyanobiont of *Stereocaulon* species have profound implications for the survival, functional ecology and evolution of the partners. We have completed the first survey of the diversity of cyanobacteria associated with this genus, which is an important component of lichen woodlands in northern ecosystems. There is apparently a deep liaison between one *Stigonema* strain and the *Stereocaulon* genus, but the exact mechanisms driving this specificity have not been assessed.

A surprising result from our analyses was the presence of several pairs of repeated and almost identical cyanobacterial sequences of both *rbc*L-X and *trn*L, in the three main clades uncovered, that are shared by different *Stereocaulon* phylospecies and separated by large distances. The mode of dispersal of symbiotic cyanobacterial strains remains unresolved. Our study poses new questions about the symbiotic association in *Stereocaulon* and presents a new experimental lichen system in Eastern North America.

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Supplementary Fig. S1. Species delimitation analyses of *Stereocaulon* accessions. A ML tree was used to evaluate three different species delimitation methods, the maximum likelihood (ML) Poisson tree process (MLPTP), a Bayesian approach to delimitate species (bPTP) and multirate approach for species delimitation (mPTP). The phylogeny on the left contained 247 sequences from GenBank and our study, with support bootstrap values on the branches. On the right, we present the results of the three analyses. The MLPTP recovered 55 species cluster, the mPTP, 35 species clusters and the bPTP, 96 species clusters. Species clusters highly supported (ML boostrap values above 90 or posterior probabilities above 0.95) for the MLPTP and bPTP methods are marked with an asterisk. We discuss in the text the most conservative approach (mPTP) and numbered all the species clusters from bottom to top (1–35).

Supplementary Table S1. Additional specimens from GenBank used for the ITS matrix and species delimitation results.

Supplementary Table S2. Additional specimens from GenBank used for the *rbc*L-X matrix.

Supplementary Table S3. Additional specimens from GenBank used for the *trn*L matrix.

Supplementary Table S4. Basic statistics and informative sites for each locus.

Supplementary Alignment S1. Alignment of the internal transcribed spacer (ITS) for 247 taxa downloaded from GenBank and our newly sequenced taxa.

Supplementary Alignment S2. Alignment of *rbc*L-*rbc*X region for 536 taxa downloaded from GenBank and our newly sequenced taxa.

Supplementary Alignment S3. Alignment of the *trn*L intron for 102 taxa downloaded from GenBank and our newly sequenced taxa.

Supplementary Alignment S4. Alignment of *rbc*L-*rbc*X region for 64 taxa downloaded from GenBank and our newly sequenced taxa.