Contrasting pattern of photobiont diversity in the Atlantic and Pacific populations of *Erioderma pedicellatum* (Pannariaceae)

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Abstract: The present study investigates the photobiont diversity of the boreal felt lichen, *Erioderma pedicellatum*. Previously sampled genetic data from Newfoundland were reanalyzed and new sequence data (16S rDNA, *rbcLX*) of the boreal felt lichen from Alaska (USA), Kamchatka (Russia), and North Trøndelag (Norway) were generated. The highest genetic diversity of the photobiont is found in Alaska and Kamchatka, indicating that these may be the primary sources of the species in the Northern Hemisphere. In Newfoundland, the photobiont of *E. pedicellatum* was screened on leaves of the symbiotic liverwort *Frullania asagrayana* and it was found to occur on trees where no other lichens were present, demonstrating that the geographical distribution, and possibly also the ecological requirement of the photobiont of *E. pedicellatum*, is wider than that of the lichen phenotype. Finally, a postulated association between the occurrence of the vegetatively reproducing *Coccocarpia palmicola* and the occurrence of the compatible photobiont of *E. pedicellatum* on the same tree could not be established.

Key words: cyanobacteria, *Frullania asagrayana*, photobiont-mediated lichen guild, *Rhizonema*, RuBisCo, tripartite interaction, 16S rDNA

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Introduction

Of all lichenized fungi, around 10% are associated with cyanobacterial photobionts as functional photosynthetic active partners that also fix atmospheric nitrogen into organic forms (Honegger 2012). According to Adams *et al.* (2013), the most common cyanobacterial photobionts are the heterocystous forms *Nostoc*, *Scytonema*, *Calothrix* and *Fischerella*. While over the last decade a

large and growing body of literature has investigated symbiotic *Nostoc* (for review, see Rikkinen 2009, 2013), the diversity of other lichenized cyanobacteria has only lately begun to be explored. A phylogenetic study by Lücking and collaborators (2009) discovered, for instance, a previously unrecognized lineage of filamentous, heterocystous cyanobacteria, which was recently named as *Rhizonema* Lücking & Barrie (Lücking *et al.* 2014). In addition, *Rhizonema* was found to be the second most diverse lichenized cyanobacterial genus after *Nostoc*.

Initially, *Rhizonema* appeared to be obligately lichenized and largely tropical, with prevalence in the recently revised basidiolichen genera *Acantholichen*, *Cora*, *Corella*, *Cyphellostereum* and *Dictyonema* (Lawrey *et al.* 2009; Yáñez *et al.* 2012; Dal-Forno *et al.* 2013, 2016; Lücking *et al.* 2014), as well as in other, unrelated but ecologically similar lichens, such as in the ascomycetous genera *Coccocarpia* and *Stereocaulon* (Lücking *et al.* 2009).

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However, our parallel study shows 1) that *Rhizonema* species are also found together with lichens from boreal forests, and 2) that they can live in symbiosis with a liverwort and not necessarily only with lichens (Cornejo & Scheidegger 2016). The present study focuses on the genetic diversity of the symbiotic *Rhizonema* species associated with the boreal felt lichen, *Erioderma pedicellatum* (Hue) P. M. Jørg.

Erioderma pedicellatum is a rare epiphytic cyanolichen, first discovered in the 19th century in New Brunswick, Atlantic Canada. This species is considered to be an ancient taxon that evolved early from a Gondwanaland stock of the genus (Jørgensen 2001). In the last 50 years, it has experienced a dramatic decline in its European and Canadian populations, and is listed as critically endangered by the International Union for the Conservation of Nature (Scheidegger 2003). In fact it was believed to be extinct in Europe by Jørgensen (1990), but young specimens were unexpectedly found in two disjunct regions in Norway (Holien *et al.* 1995; Reiso & Hofton 2006), apparently indicating recolonization. More recently, *E. pedicellatum* was surprisingly discovered in rich populations in damp conifer forests in Alaska (Nelson *et al.* 2009) and Kamchatka (Stepanchikova & Himelbrant 2012), confirming its restriction to moist boreal forests, while other species of the genus have mainly evolved in South America and South-East Asia (Jørgensen 2001; Jørgensen & Arvidsson 2002; Jørgensen & Sipman 2002).

During recent decades, a number of studies have investigated habitat requirements and environmental effects that threaten the existence of *E. pedicellatum*. Different causes for the decline of this lichen are well documented, for example, forestry activities in Sweden (Ahlner 1954). In Maritime Canada, the loss of thalli due to wind exposure after clear-cut, acid rain, and air pollution, are likely to contribute to the rapid population decline (Maass & Yetman 2002; Richardson & Cameron 2004; Cameron *et al.* 2010, 2013a, b). Less is known, however, about the life cycle of *E. pedicellatum* and the resynthesis of the lichen symbiosis. Like all

sexually reproducing lichen species, a germinating ascospore of *E. pedicellatum* will not be able to form a lichen thallus until it makes contact with a compatible photosynthetic cyanobiont. Among other reasons, it is quite conceivable that populations of *E. pedicellatum* in Maritime Canada are declining due to the limited availability of the photobiont in natural habitats. In other words, fungal hyphae of *E. pedicellatum* would only rarely find a suitable photobiont in order to re-establish the lichen symbiosis.

Many fungal species have been shown to associate with more than one cyanobiont, and these are often also shared by several other cohabiting cyanolichen species (for a review, see Rikkinen 2009, 2013, 2015). Our previous investigation at the regional scale in Newfoundland has shown, however, that *E. pedicellatum* is highly selective towards one *Rhizonema* strain (Cornejo & Scheidegger 2016). Additionally, this study reports that various closely related *Rhizonema* form symbioses with ecologically similar lichen species, but from different genera, such as an undetermined *Lichinodium* Nyl. species, *Moelleropsis nebulosa* subsp. *frullaniae* Maass, and *Parmeliella parvula* P. M. Jørg. Based on these initial findings, the present study also explores whether the boreal felt lichen remains highly selective towards the same *Rhizonema* strain throughout the whole distribution range on the Atlantic and Pacific Coasts.

With respect to the photobiont, Jørgensen *et al.* (2009) assumed the presence of a photobiont-mediated lichen guild, *Coccocarpia-Erioderma*, for *E. mollissimum* (Sampaio) Du Rietz and *E. pedicellatum* in forests of Newfoundland, according to Rikkinen (2002, 2003). A recent investigation confirmed this assumption, demonstrating that *E. pedicellatum* shares an identical *Rhizonema rbcLX* haplotype with local populations of the core species *Coccocarpia palmicola* (Spreng.) Arv. & D. J. Galloway, and with cyanobacterial colonies on the co-occurring liverwort *Frullania asagrayana* Mont. (Cornejo & Scheidegger 2016). Nevertheless, the question still remains whether the compatible photobiont can be found within suitable, as yet uncolonized habitats of

E. pedicellatum. According to the concept of photobiont-mediated lichen guilds, the photobiont of *E. pedicellatum* is hypothesized to be more frequent on trees colonized by vegetatively reproducing lichens that share the same photobiont, than on trees without these core lichen species. However, our previous study (Cornejo & Scheidegger 2016) demonstrated that in Newfoundland, a cyanobacteria-mediated tripartite interaction between *Rhizonema*, *F. asagrayana* and lichenized fungi must be taken into consideration. Therefore, one of the purposes of this study is to test whether the compatible *Rhizonema* is present in uncolonized habitats of *E. pedicellatum*, by screening for this cyanobacterium in leaves of *F. asagrayana*, collected from trees with and without the core lichen species *C. palmicola*.

The most significant threats to lichens in boreal forests today are the loss of mature trees, the disappearance of old-growth forests, and changes in tree species composition. Therefore, this study attempts to gain a better understanding of the boreal felt lichen, and to contribute to its continuous persistence in boreal forests. Partial sequences of both the small subunit rDNA gene (16S) and the RuBisCo region *rbcL-rbcX* (*rbcLX*) were sampled to identify locally restricted cyanobacterial clusters. This paper reports on the surprising findings that the specific photobiont of *E. pedicellatum* is widespread in uncolonized habitats in Newfoundland and that the highest photobiont diversity of *E. pedicellatum* is found in populations on the Pacific coast.

Materials and Methods

Collection sites of *Erioderma pedicellatum*

Specimens of *E. pedicellatum* were collected in Alaska (USA) and the Kamchatka Peninsula (Russia) (Fig. 1). In addition, a small fragment of *E. pedicellatum* from North Trøndelag (Norway) was kindly provided by the TRH herbarium for the purpose of this study (Fig. 2B). A humid continental climate, greatly influenced by the sea, and coniferous forests characterize all collection sites where *E. pedicellatum* grows predominantly on the bark of conifers: in Alaska on *Picea glauca* (Moench) Voss., in Kamchatka on *Picea ajanensis* (Lindl. et Gord.) Fisch. ex Carr., and in Europe on *Picea abies* (L.) H. Karst.

Specimens from south-central Alaska were collected in the Denali State Park (DSP) and the Denali National Park and Preserve (DNPP), located between 62°18'–64°04' N latitude and 152°52'–148°48' W longitude (for sampling details, see Nelson *et al.* 2009). In Kamchatka, specimens were collected in August 2009 during a collecting trip in the Kronotsky Nature Reserve, south of the Nikolka Volcano, close to the confluence of the Levaja Schapina and Ipuin rivers. One collection site was 2.2 km to the north of the mouth of the Ipuin River at 300 m altitude. Two other locations were c. 2.5 km, and the last 3.5 km to the north-east of the mouth of this river, at 331, 350, and 370 m altitude, respectively. The vegetation of these sites was an pristine *Picea ajanensis* forest with green mosses on the slopes. The collection area ranged between 55°08'07"–38" N latitude and 159°57'23"–159°59'24" E longitude (Stepanchikova & Himelbrant 2012).

Finally, this study utilizes molecular data of specimens collected in Newfoundland and analyzed in a previous study (Cornejo & Scheidegger 2016). In Newfoundland, *E. pedicellatum* grows mainly on *Abies balsamea* (L.) Mill. One collection site was on the southern coast of Newfoundland, in Bay D'Espoir (BDE) (47°54'22.3194" N, 55°34'37.74" W) (Fig. 1), while all others were in the Avalon Peninsula, in the vicinity of Lockyers Waters (LW), Fourth Pond (FP), Sandy Pond (SP), Long Harbour (LH), and Placentia (P), between 47°25'–47°14' N latitude and 53°57'–53°16' W longitude (Fig. 1).

Study species

This work studies species of the lichen guild *Coccocarpia-Erioderma* collected in Newfoundland, that is, *C. palmicola*, *E. pedicellatum*, *Lichinodium* sp., *Moelleropsis nebulosa* ssp. *frullaniae*, and *Parmeliella parvula*, as well as the liverwort *Frullania asagrayana* (Table 1). Other lichen species, such as *Pectenia plumbea* (Lightf.) P. M. Jørg. *et al.*, *Lobaria pulmonaria* (L.) Hoffm., *Lobaria scrobiculata* Nyl. ex Croub., *Fuscopannaria ahlneri* P. M. Jørg., *Peltigera collina* (Ach.) Röhl., and *Pseudocyphellaria crocata* (L.) Vain., share the same habitat but are already known to associate with *Nostoc* strains (Miadlikowska & Lutzoni 2000; Stenroos *et al.* 2003; Wedin *et al.* 2009). Thus only some of these species were included in the dataset as outgroup taxa.

In contrast, *Erioderma sorediatum* P. M. Jørg. & D. J. Galloway and an undefined *Leptogidium* Nyl. species are reported to associate with *Scytonema* strains (Jørgensen 2006; Muggia *et al.* 2011). According to Lücking *et al.* (2009), lichenized *Scytonema*-like cyanobacteria might belong to *Rhizonema*. Consequently, specimens of *E. sorediatum* and *Leptogidium* sp. from the Tongass National Forest in south Alaska were added to the dataset to test whether their photobionts are also *Rhizonema* species. To minimize population damage due to sampling, only fragments of *E. pedicellatum* were harvested (Fig. 2). The specimen vouchers and GenBank accession numbers of loci sampled are listed in Table 2.

TABLE 1. *Taxa and number of specimens used in this study.*

Taxon	* Locality and specimen collector(s)	Sampling note	Number of specimens
<i>Coccocarpia</i>	Newfoundland, C. Scheidegger		13
<i>palmicola</i>	Brazil, C. Cornejo, C. Scheidegger		3
<i>Erioderma pedicellatum</i>	Alaska, P. Nelson & C. Scheidegger		12
	Newfoundland, C. Scheidegger		19
	Kamchatka, I. Stepanchikova & D. Himelbrant		8
	Norway, G. Gaarder, TRH Herbarium	TRH L-27011	1
	Alaska, K. Dillman		1
<i>E. sorediatum</i>	Newfoundland, C. Scheidegger	Trees without lichens	13
<i>Frullania asagrayana</i>		Only <i>C. palmicola</i> on trees	13
		<i>C. palmicola</i> and <i>E. pedicellatum</i> on trees	7
		<i>P. parvula</i> and <i>Lichinodium</i> sp. on tree	1
			1
<i>Leptogidium</i> sp.	Alaska, K. Dillman		10
<i>Lichinodium</i> sp.	Newfoundland, C. Scheidegger		2
<i>Moelleropsis nebulosa</i> ssp. <i>frullaniae</i>	Newfoundland, C. Scheidegger		12
<i>Parmeliella parvula</i>	New Zealand, Stenroos et al. (2006)	GenBank: DQ265951 and DQ266030	1
<i>Stereocaulon fronduliferum</i>			

* Majority of specimens from Newfoundland collected for previous study (Cornejo & Scheidegger 2016).

Sampling design for *Frullania asagrayana*

In order to verify the presence of the compatible photobiont of *E. pedicellatum* in potential habitats of this species in Newfoundland, we collected *F. asagrayana*, colonized by cyanobacterial mats in two *E. pedicellatum* localities, namely Fourth Pond and Lockyers Waters, where 31 liverwort specimens were randomly sampled from trees: 1) without lichens, 2) where *C. palmicola* was present, and 3) where *C. palmicola* and *E. pedicellatum* were present on trees. Three additional *F. asagrayana* specimens from the Sandy Pond locality were found on trees that were already colonized by *C. palmicola* and *E. pedicellatum* or by *P. parvula* and *Lichinodium* sp. In total, 34 specimens of *F. asagrayana* were collected and prepared for DNA extraction (Table 3). The null hypothesis here was that there is no association between the occurrence of *C. palmicola* and the occurrence of the compatible *Rhizonema* on the same tree, while the alternative hypothesis assumes that the compatible *Rhizonema* is found in a higher proportion of trees where *C. palmicola* is present. A one-sided test was chosen because we expected that the presence of the core lichen *C. palmicola* supports the presence of the

compatible *Rhizonema* on the same tree, according to the concept of the photobiont-mediated lichen guild (Rikkinen 2002). Fisher’s exact test (R 3.2.2, R Core Team 2014) used a 2 × 2 contingency table for the test of the null hypothesis and it was applied with a significance level of $P = 0.05$.

Laboratory procedures and data analyses

DNA isolation and genetic markers

The DNA isolation of all specimens, the polymerase chain reactions of the 16S and *rbclX* loci, the cycle sequencing, and sequence alignments were performed as described in Cornejo & Scheidegger (2016). For the amplification of shorter fragments with higher *Rhizonema* specificity, internal primer pairs were designed (16S: CYA-16S-F 5'-ctagtgtgtagggtaaaagctt-3' and CYA-16S-R 5'-taccgcactctagcttga-3'; *rbclX*: CYA-*rbclX*-F 5'-atttggtggtggtactttgggt-3' and CYA-*rbclX*-R 5'-agccaaagtgcctaaggagaat-3') based on sequences obtained with the broad range primers CYA106F–CYA781R (Nübel et al. 1997) and CW–CX (Rudi et al. 1998), following the author’s PCR instructions.

FIG. 1. Circumpolar map showing sampling locations for *Erioderma pedicellatum*. Collection sites in Newfoundland include site a, close to the Bay D’Espoir (one site) and site b, Avalon Peninsula (all other specimens). Pie charts show the distribution of 16S and *rbclX* haplotypes found in each location. The size of circles is directly proportional to the number of individuals analyzed. The ERI/02-individual from Norway belongs to the 16S R3-haplotype and is not shown. A full list of haplotypes per collection site can be found in Table 2. (Circumpolar map: Wikimedia Commons; Avalon Peninsula: artofanderson.com)

FIG. 2. *Erioderma pedicellatum*. A, thallus showing many apothecia; B, sampled piece of thallus (ERI/02 from Norway). A wound on the upper surface of the thallus exposes the blue-greenish, cyanobacterial layer (arrow). Scale = c. 1 mm

DNA polymorphism of sampled 16S and rbcLX sequences

To eliminate ambiguously aligned fragments, both datasets were aligned using Gblocks (Castresana 2000;

Talavera & Castresana 2007) on the Phylogeny.fr platform (Dereeper *et al.* 2008, 2010). Within Gblocks, the options “smaller final blocks, gaps position on the final blocks, and fewer strict flanking positions” were used.

TABLE 2. Vouchers of sampled lichen specimens, their *rbcLX* and 16S haplotypes and GenBank accession numbers.

Taxon	WSL-voucher*	Country	Collection site†	<i>rbcLX</i> haplotype	16S haplotype	GenBank accession numbers	
						<i>rbcLX</i>	16S
<i>Coccocarpia palmicola</i>	SC/67A	Canada	LH	—	R1	—	KT884015
	X6	Brazil	SP	R2	R2	KT867550	KT884016
	X18	Brazil	RJ	R2	R2	KT867551	KT884017
	X19	Brazil	RJ	R2	R2	KT867552	KT884018
<i>Erioderma pedicellatum</i>	BDE19	Canada	BDE	—	R1	—	KT884019
	ERI/01a	USA	DNPP	R3b	R3	KT867538	KT884020
	ERI/01b	USA	DNPP	R3b	R3	KT867539	KT884021
	ERI/01c	USA	DNPP	—	R3	—	KT884022
	ERI/01d	USA	DNPP	R3a	R3	KT867540	KT884023
	ERI/01e	USA	DNPP	R3b	R3	KT867541	KT884024
	ERI/02	Norway	TR	—	R3	—	KT884025
	ERI/06	USA	DSP	R3b	R3	KT867542	KT884026
	ERI/07	USA	DSP	R3b	R3	KT867543	KT884027
	ERI/26	USA	DSP	R1	R1	KT867544	KT884028
	ERI/28	USA	DNPP	—	R3	—	KT884029
	ERI/29	USA	DNPP	R3b	R3	KT867545	KT884030
	ERI/30	USA	DNPP	R3b	—	KT867546	—
	ERI/31	USA	DSP	R3b	R3	KT867547	KT884031
	ERI/32	USA	DSP	—	R3	—	KT884032
	ERI/33b	USA	DSP	—	R3	—	KT884033
	ERI/33c	USA	DSP	—	R3	—	KT884034
	ERI/33i	USA	DSP	—	R3	—	KT884035
	ERI/33j	USA	DSP	—	R3	—	KT884036
	ERI/34	USA	DSP	R3b	R3	KT867548	KT884037
	ERI/35a	USA	DSP	R3b	R3	KT867549	—
	X168	Russia	K	—	R3	—	KT884038
	X169	Russia	K	R3b	R3	KT867553	KT884039
	X170	Russia	K	R1	R1	KT867554	KT884040
	X171a	Russia	K	—	R3	—	KT884041
	X171b	Russia	K	—	R3	—	KT884042
	X171c	Russia	K	R1	—	KT867555	—
	X172	Russia	K	R5a	—	KT867556	—
	X174	Russia	K	—	R5b	—	KT884043
<i>E. sorediatum</i>	ERI/04a	USA	TNP	—	R6	—	KT884044
<i>Leptogidium</i> sp.	ERI/04b	USA	TNP	—	R7	—	KT884045
<i>Lichinodium</i> sp.	SC/69	Canada	LH	—	R3	—	KT884046
<i>Parmeliella parvula</i>	SC/68	Canada	LH	—	R6	—	KT884048
	SC/86B	Canada	LH	—	R6	—	KT884049

* All specimens are stored frozen at WSL (C. Scheidegger-collection), except BDE19.
† Collection site key: Brazil: SP = State Paraná; RJ = Rio de Janeiro. Canada-Newfoundland: BDE = Bay D’Espoir; LH = Long Harbour; LW = Lockyer’s Waters. Norway: TR = North Trøndelag. Russia: K = Kamchatka. USA-Alaska: TNP = Tongass National Park; DSP = Denali State Park; DNPP = Denali National Park and Preserve.

DnaSP v.5.10.01 (Librado & Rozas 2009) was used for DNA polymorphism analyses, using options for haploid prokaryotes and excluding sites with gaps. Analyses focused on haplotype diversity (Hd, probability that two randomly chosen haplotypes are different within the sample) (Nei & Tajima 1983), the pairwise estimates of nucleotide divergence (Pi, probability that two randomly taken nucleotides from the same position are different) (Jukes & Cantor 1969), and the average of nucleotide differences (K). In order to test the null hypothesis that sequences are evolving according to neutral expectations, Tajima’s *D*-test were performed on each locus (Tajima 1989). Signal of recombination was sorted on each locus using the Phi test (Bruen *et al.* 2006) as applied in the software SplitsTree4 (v.4.11.3; Huson & Bryant 2006), since DnaSP uses a four-gametic statistic whose infinite sites assumption can be violated by the high mutation rate of bacteria.

TABLE 3. Screening of *Rhizonema* strains on specimens of the liverwort *Frullania asagrayana* from Newfoundland. The presence (+) or absence (–) of lichen taxa on sampled trees, and the *rbcLX* type found in the liverwort are indicated.

Locality	Specimen ID	Ep on tree	Cp on tree	<i>rbcLX</i> type*
Sandy Pond	SC/41A_Fru [†]	–	–	R6a
	SC/47B_Fru	–	+	R1
	SC/58_Fru	–	+	R1
Fourth Pond	Fru1	–	–	No PCR
	Fru2	–	–	No PCR
	Fru3	–	–	R1
	Fru4	–	–	R3a
	Fru5	–	–	R1
	Fru6	–	–	R1
	Fru7	–	–	R6c
	Fru8 [‡]	–	–	Ep & Lsp
	Fru9	–	–	No PCR
	Fru10 [‡]	–	–	Ep & Pp
	Fru11	–	+	R1
	Fru12	–	+	R6a
	Fru13	–	+	R1
	Fru14 [‡]	–	+	Ep & Pp
	Fru15	–	+	R6a
Lockyers Waters	Fru16	–	+	R1
	Fru17 [‡]	–	+	Ep or Pp
	Fru18 [‡]	–	+	Ep & Pp
	Fru19	–	+	R1
	Fru20	–	+	R1
	Fru21	+	+	R1
	Fru22	+	+	R1
	Fru23	+	+	R1
	Fru24	+	+	R1
	Fru25	+	+	R1
	Fru28	+	+	R6a
	Fru29	+	+	R6a
	Fru30	–	–	R1
	Fru31	–	–	No PCR
	Fru32	–	–	R1
	Fru33 [‡]	–	+	Ep & Pp

* GenBank accession numbers KT867586–KT867609 (Cornejo & Scheidegger 2016).

[†] Non-systematically sampled tree, SC/41A, with *P. parvula* and *Lichinodium* sp. on tree.

[‡] Dimorphic *rbcLX* sequences, representing two different *Rhizonema* strains. These sequences were excluded from haplotype analysis, hence they are not designated with a haplotype abbreviation but with both lichen names to which sequences correspond.

Cp = *C. palmicola*; Ep = *E. pedicellatum*; Lsp = *Lichinodium* sp.; Pp = *P. parvula*

Haplotype network

The statistical parsimony algorithm applied in TCS v.1.21 (Clement *et al.* 2000) was used to construct a haplotype network that estimates 1) gene genealogies,

including multifurcations and/or reticulations, and 2) the species-subspecies interface, using a 95% connection limit and treating gaps as missing data. In a haplotype network, the aim is to represent all equally most-parsimonious solutions, and missing data are likely to result in greater network complexity (Joly *et al.* 2007). Therefore, the datasets obtained from Gblocks, which excluded all ambiguous sites from the matrix, were used for TCS haplotype analyses. Based on Hart & Sunday (2007), the present study considered unconnected subnetworks as a general tool in species assignment. Haplotypes were hierarchically nested to visualize higher-order patterns of association and colour-coded using the graphic software Omnigraffle 5.4.4 (Omni Group, Seattle, USA).

Results

Primer specificity, dimorphic sequences and alignments

Out of the eight specimens of *E. pedicellatum* from Kamchatka, six 16S and four *rbcLX* sequences were obtained, since the others did not amplify with the internal primers, and generated several PCR products with the external primers. Similarly, only the 16S sequences of the specimens ERI/02 (*E. pedicellatum* from Norway), *E. soreliatum* and *Leptogidium* sp. were obtained. Consequently, these samples are not represented in the *rbcLX* haplotype analysis. Furthermore, four DNA samples of *F. asagrayana* did not amplify with any *rbcLX* primers (Table 3), and most specimens of this liverwort gave very low PCR concentrations with the 16S-primers. In this case, a reamplification with internal primers has not been successful and the few sequences obtained were mostly dimorphic. For this reason, 16S-sequences of *F. asagrayana* were not used for the present study.

Six samples of *F. asagrayana* were excluded from analyses because their sequences contained some dimorphic nucleotide sites. A separate analysis of dimorphic sites demonstrated that there were two possible DNA sequences in each sequence belonging to the *Rhizonema* strains under investigation (Table 3). In other words, different strains of lichen-compatible cyanobacteria were found that belong to the genus *Rhizonema* and live epiphytically on the same liverwort leaf. In five cases, the photobionts of *E. pedicellatum*

and *P. parvula* were detected on the same branch. Further, the cyanobacterial partners of *E. pedicellatum* and *Lichinodium* sp. were also found to share the habitat on a single branch of the liverwort.

Finally, the present study used data from 116 specimens that included one liverwort species and eight lichen species (Table 1). The 16S partial locus of 35 samples and the *rbcLX* region of 20 specimens were generated (Tables 2 & 3) and analyzed together with 42 16S and 76 *rbcLX* sequences produced previously (Cornejo & Scheidegger 2016), as well as the sequences DQ265951 (16S) and DQ266030 (*rbcLX*) acquired from GenBank. Gblocks calculated no ambiguous positions (i.e., 100% conserved) for the 573 bp alignment of the 16S dataset, including three sequences with each single gap. However, some *rbcLX* sequences were amplified with external primers and others with internal primers, resulting in longer and shorter *rbcLX* sequences. Consequently, 375 positions (57%) of the *rbcLX* alignment were considered conserved by Gblocks, including 17 sequences containing one gap, one sequence with two gaps, two with 32, and one sequence with 34 gaps.

Screening of the compatible *Rhizonema* strain of *E. pedicellatum*

From 34 samples of *F. asagrayana*, four gave no PCR product and one was on a tree colonized by *P. parvula* and *Lichinodium* sp. but not *C. palmicola* or *E. pedicellatum* (Table 3). These five specimens were therefore excluded from further analysis. The photobiont of *E. pedicellatum* was present in 23 (79.3%) out of the 29 remaining samples, including six dimorphic *rbcLX* sequences. Out of 20 samples that were collected from trees where either *E. pedicellatum* or *C. palmicola* or both were present, 16 (80%) samples contained the *E. pedicellatum*-specific *Rhizonema*. Furthermore, out of the 9 samples that were collected from trees where neither of the two lichen species was present, 7 (77.7%) contained the *E. pedicellatum*-specific *Rhizonema*. The null hypothesis, which assumed a lack of

association between the occurrence of the compatible *Rhizonema* and the presence of *C. palmicola* on the same tree, failed to be rejected. Thus, no significant difference was found between trees without lichens and i) trees with *C. palmicola* ($P = 0.5504$), ii) trees with *C. palmicola* and *E. pedicellatum* ($P = 0.6077$), and iii) trees with *E. pedicellatum* and *C. palmicola* or *C. palmicola* alone on the same tree ($P = 0.6247$) (Table 4).

16S and *rbcLX* sequence variation

In both datasets polymorphism was detected (Table 5). However, polymorphism within *Rhizonema* of the same lichen species was rare. In Newfoundland, within-host polymorphism was only detected in *F. asagrayana* and *P. parvula*. *Rhizonema* of *E. pedicellatum* were polymorphic only in Alaska and Kamchatka (Fig. 1). Similarly, polymorphism was only detected between Brazilian and Canadian specimens of *C. palmicola* but not within populations. *Rhizonema* of *E. pedicellatum* in Newfoundland was monomorphic for both loci (Fig. 1), whereas strains from Kamchatka showed the highest nucleotide diversity per site (Π) and average of nucleotide differences (K) of all *Rhizonema* strains studied (Table 5).

Within-locus analyses (78 16S-sequences of eight lichen species and 96 *rbcLX*-sequences of six lichen and one liverwort species) gave a haplotype diversity of 6 and 9 haplotypes, respectively. But while both regions obtained similar values for the haplotype diversity (H_d), the nucleotide diversity (Π) showed a significant difference between both loci, the Π value of the 16S region being around ten times lower than that of the *rbcLX* locus (Table 5). Of all variable sites within both datasets, only one consisted of two nucleotides next to each other; all others were SNPs (single-nucleotide polymorphisms). The largest number of SNPs was found within the intergenic spacer of the *rbcLX* region, then within the partial *rbcL* coding region, whereas the partial *rbcX* region was monomorphic among the specimens studied (data not shown). Selective

TABLE 4. Evaluation of the *Rhizonema rbcLX* haplotype (R1) found in *F. asagrayana* and compatible with *E. pedicellatum* in Newfoundland, using 2 × 2 contingency tables for the observed presence (+) or absence (–) of the R1. The P-values indicate results of one-tailed Fisher’s exact tests for a small sample size.

Item	Sampling characteristic	Number of specimens	Number of Successful PCR’s	2 × 2 contingency table		P-value
				R1 (+)	R1 (–)	
A	*Cp on tree	13	13	11	2	0.5504
	No lichen on tree	13	9	7	2	
	Total			18	4	
B	Cp and Ep on tree	7	7	5	2	0.6077
	No lichen on tree	13	9	7	2	
	Total			12	4	
C	Cp and Ep, or Cp alone on tree	20	20	16	4	0.6247
	No lichen on tree	13	9	7	2	
	Total			23	6	

*Cp = *Coccocarpia palmicola*; Ep = *Erioderma pedicellatum*.

TABLE 5. Summary of the nucleotide and haplotype diversity of polymorphic *Rhizonema* as calculated by DnaSP. Within-host polymorphism was found only in 16S-sequences of *E. pedicellatum* from Alaska and Kamchatka, and in *rbcLX*-sequences of *E. pedicellatum* (Alaska and Kamchatka), *P. parvula* and *F. asagrayana*.

	Partial 16S*			Partial <i>rbcLX</i> *				
	All	<i>E. pedicellatum</i>		All	<i>E. pedicellatum</i>		<i>P. parvula</i>	<i>F. asagrayana</i>
		Alaska	Kamchatka		Alaska	Kamchatka	Newfoundland	
Number of sequences	78	17	6	96	12	4	11	24
Variable sites	4	3	5	25	9	13	6	17
Parsimony informative sites	3	0	3	22	1	3	2	14
Total number of mutations, Eta	4	3	5	27	9	14	6	17
Number of segregating sites (S)	4	3	5	25	9	13	6	18
Number of haplotypes (H)	6	2	3	9	3	3	3	4
Haplotype diversity (Hd)	0.692	0.118	0.600	0.691	0.318	0.833	0.345	0.471
Variance of haplotype diversity	0.00064	0.01023	0.04630	0.00172	0.02680	0.04948	0.02967	0.01104
Nucleotide diversity per site (Pi)	0.00195	0.00062	0.00396	0.01861	0.00436	0.02151	0.00369	0.01698
Average of nucleotide differences (K)	1.09957	0.35294	2.26667	6.30746	1.63636	7.33333	1.38182	5.80797

* Alignment length of 16S: 1–573 nu; *rbcLX*: 1–375 nu.

neutrality was examined by Tajima’s method, which tests the standard neutral model for a given region of DNA sequence (Tajima 1989). Tajima’s *D* showed values close to zero (16S: *D* = 0.73517; *rbcLX*: *D* = 0.60414) and were not significant (*P* > 0.10), suggesting that both 16S and *rbcLX* regions are apparently free from strong selective pressure. Furthermore, the Phi test (SplitsTree4) found no statistically significant evidence for recombination within the 16S (*P* = 0.793) or *rbcLX* matrix (*P* = 0.066).

Haplotype analyses

At 95% statistical parsimony, eight connection steps were calculated for the *rbcLX* dataset, resulting in two subnets (Fig. 3). The 96 analyzed *Rhizonema* sequences contained nine diverging *rbcLX* haplotypes in accordance with the DnaSP number of haplotypes (H). Subnet 1 is dominated by the haplotype R1, which includes specimens of *C. palmicola*, *E. pedicellatum* and *F. asagrayana* that were sampled in Newfoundland, as well as one specimen of *E. pedicellatum* from Alaska (ERI/26)

FIG. 3. Two haplotype analyses of relationships among symbiotic *Rhizonema*. Left: two unconnected subnets representing 96 *rbcLX* sequences of symbiotic *Rhizonema* associated with lichens and a liverwort. Right: one network representing 78 16S sequences of symbiotic *Rhizonema* associated with lichens. Ovals or segments are coloured according to the symbiotic partner. Number of specimens per symbiotic partner is adjacent to segments; letters in parentheses indicate origin of specimens. Key to countries: A, Alaska; Br, Brazil; K, Kamchatka; N, Norway; NF, Newfoundland; NZ, New Zealand.

and two from Kamchatka (X170, X171c). Most *Rhizonema* sequences of *E. pedicellatum* from Alaska are found in R3b, one specimen from Kamchatka (X169) being the exception within R3b. On the other hand, all *Rhizonema* samples of *Lichinodium* sp. are included in R3a, one specimen of *E. pedicellatum* from Alaska (ERI/01d) being the exception within R3a. Other haplotypes within subnet 1 are R2 (comprising three Brazilian specimens of *C. palmicola*), R4 (comprising both specimens of *M. nebulosa* ssp. *frullaniae* from Newfoundland), and R5a (specimen X172 of *E. pedicellatum* from Kamchatka). Finally, in *P. parvula* from Newfoundland, three distinct *rbcLX* haplotypes (R6a–c) were found plotted in subnet 2. Interestingly, the sequence DQ266030 of *S. fronduliferum* from New Zealand shares the same *rbcLX* haplotype, R6a, as specimens of *P. parvula* from Newfoundland.

The quantity of *rbcLX* haplotypes varied from species to species. Four different types (R1, R3a, R3b and R5a) were found within 32 samples of *E. pedicellatum* (Table 1), two (R1 and R2) in 16 specimens of *C. palmicola*, and three in 11 specimens of *P. parvula* (R6a, R6b and R6c). However, in each case (ten *Lichinodium* specimens (R3a) and two individuals of *M. nebulosa* ssp. *frullaniae* (R4)) only one haplotype was found. This result may reflect differences in the number of individuals sampled, considering the huge difference between 32 individuals of *E. pedicellatum* and only two of *M. nebulosa* ssp. *frullaniae*. However, it also shows an important discrepancy between specimens from Newfoundland with similar sampling size: three different haplotypes in *P. parvula* ($n = 11$) but only one in each of *C. palmicola* ($n = 13$) and *Lichinodium* sp. ($n = 10$).

The distribution of *rbcLX* haplotypes was also heterogeneous depending on the collection site. Specimens of *C. palmicola* from Brazil (R2) differed from the *rbcLX* haplotype found in Newfoundland of the same species (R1). Out of 16 specimens of *E. pedicellatum* from Newfoundland, one *rbcLX* haplotype (R1) was found, while three were detected out of 12 specimens from Alaska (R1, R3a and R3b) and three out of four specimens from Kamchatka (R1, R3b and R5a). On the other hand, all 11 analyzed specimens of *P. parvula* originate from Newfoundland, and are associated with three different *rbcLX* haplotypes.

In contrast to the *rbcLX* matrix, the 16S dataset did not contain specimens of *F. asagrayana* (Fig. 3). At the 95% parsimony limit, seven haplotypes were found that were separated by a maximum of ten connection steps. Thus TCS analysis resulted in one haplotype more than the DnaSP haplotype calculation, probably due to differences in the handling of gaps; while DnaSP excluded gaps from pairwise comparisons, TCS treated them as missing data. TCS assigned the highest outgroup probability to the R1-haplotype for the *rbcLX* subnet 1, and to the R6a-haplotype for the subnet 2. For the 16S-network, it calculated the highest outgroup probability for the R3-haplotype. Since haplotypes were hierarchically nested, the arrangement of haplotypes was somehow different for both analyses. Even though the 16S-network showed less-differentiated haplotypes, the outcome produced basically the same patterns as the *rbcLX* networks. Less differentiation was found within the haplotype R3, which contained specimens of *E. pedicellatum* from Alaska, the specimen ERI/02 from Norway and four specimens from Kamchatka, as well as all specimens of a *Lichinodium* species from Newfoundland. Similarly, all specimens of *P. parvula* shared one haplotype (R6) together with *S. fronduliferum* from New Zealand and the 16S-sequence of *E. soreliatum* from south Alaska. In contrast, the 16S-sample of a *Leptogidium* species from south Alaska resulted in the different haplotype R7.

Discussion

Molecular techniques offer a highly practical way to study the diversity of symbiotic cyanobacteria since the few morphological structures of these organisms are limited and tend to become modified within the lichen thallus. Since the initial description of the genus *Rhizonema* (Lücking *et al.* 2009, 2014), this work and our parallel study (Cornejo & Scheidegger 2016) are the first dedicated to the genetic diversity within this genus. This study shows the wide geographical distribution of *Rhizonema* strains in boreal forests, associated with a high genetic diversity and their occurrence as the photobiont of many different lichen species. For instance, *Rhizonema* strains were identified which associate with *E. soreliatum* and an undetermined *Leptogidium* species. Only recently, Muggia *et al.* (2011) recovered the genus *Leptogidium* for “*Scytonema*-containing” species, which are morphologically similar to the convergently evolved genus *Polychidium*. Findings from the present study indicate that *Leptogidium* may associate with *Rhizonema* instead of *Scytonema*, and phylogenetic study of the mycobiont and photobiont of *Leptogidium* specimens may provide clarity on this issue. Similarly, this study shows that at least two species of *Erioderma* associate with *Rhizonema*, instead of *Scytonema*. Lücking *et al.* (2009) showed that *Rhizonema* strains are widely distributed in tropical regions and a more comprehensive dataset of the genus *Erioderma* might confirm the association with *Rhizonema* for the other taxa as well.

Genetic diversity of *Rhizonema*

Within-locus calculations of the nucleotide diversity (π ; Table 5), including all species studied, showed a ten times higher nucleotide diversity for *rbcLX* data, resulting in higher haplotype diversity and, consequently, higher resolution power in phylogenetic analysis for this locus (Fig. 3). For example, the *rbcLX* haplotype analysis revealed two *Rhizonema* species, indicated by two unconnected subnets (Fig. 3; Hart & Sunday 2007). Tajima’s statistics suggested

that both 16S and *rbcLX* loci of *Rhizonema* have been undergoing stable history. Additionally, there was no signal of recombination between the specimens studied. However, in the present work, recombination could also be underestimated as a consequence of the limited amount of loci analyzed, the moderate sequence length for each analyzed locus, and the limited divergence between sequences involved in recombination events, particularly evidenced by the low nucleotide diversity (Π) found within the 16S region. Furthermore, most within-host sequences were monomorphic, indicating a high level of clonal reproduction. Larger sampling size (specimens and nucleotide sites) from highly polymorphic *Rhizonema* from Alaska or Kamchatka, for instance, could provide clearer evidence of recombination.

In Newfoundland, *Rhizonema* strains were divided by their host affiliation, except those strains that were detected in *F. asagrayana* and *P. parvula* with the *rbcLX* locus. Both species can associate with several different haplotypes ($H = 4$ or 3 , respectively). *Frullania asagrayana* also presented similar Π -values to those of the complete dataset (Table 5). *Rhizonema* of *E. pedicellatum* from Kamchatka showed the highest nucleotide diversity per site (*rbcLX*- Π : 0.02151) out of all *Rhizonema* strains studied, which was around three times higher than the nucleotide diversity found in *Nostoc* of the sexually reproducing *Pectenia plumbea* within the same locus (Otálora et al. 2013). It is also interesting because three *Rhizonema* haplotypes of *E. pedicellatum* from Kamchatka contained c. 20% more nucleotide diversity than four *Rhizonema* haplotypes of *F. asagrayana* from Newfoundland. In contrast, each *E. pedicellatum*, *C. palmicola*, and a *Lichinodium* species from Newfoundland presented monomorphic DNA structure. This finding indicates clonal reproduction patterns for these *Rhizonema* within a restricted regional scale, suggesting vegetative reproduction of the lichen linked to vertical transmission of the photobiont. Vegetative reproduction is indeed the case in *C. palmicola* and *Lichinodium* sp., but not in *E. pedicellatum*.

The *Erioderma pedicellatum*–*Rhizonema* association

Although *E. pedicellatum* must always begin the life cycle starting from ascospores, this study detected only the *rbcLX* haplotype R1 in Newfoundland. This tight association between symbionts is rather expected for vegetatively-reproducing lichen species where the mycobiont and photobiont are vertically transmitted to the next generation (Otálora et al. 2010, 2013; Fernández-Mendoza et al. 2011; Dal Grande et al. 2012; O'Brien et al. 2013). Furthermore, R1 was found to be the most common *Rhizonema* haplotype, which is also present in Alaska and Kamchatka with a long distribution range within circumboreal forests. Considering that both datasets are hardly different, the study found four haplotypes in total for *E. pedicellatum* within the *rbcLX* matrix, from which R1, R3b and R5a were found in Kamchatka, and R1, R3a and R3b in Alaska. Similarly, of three haplotypes within the 16S matrix, R1, R3 and R5b were found in Kamchatka. This pattern demonstrates that the geographical limitation of the *E. pedicellatum* photobiont is true for Newfoundland, but not for Alaska or Kamchatka, where *E. pedicellatum* associates with some *Rhizonema* haplotypes that have a global distribution and are closely related to strains found in unrelated, tropical lichens.

Interestingly, our study suggests *E. pedicellatum* is a generalist with respect to photobiont association, at least in Alaska and Kamchatka. This is the case if a fungus associates with more than one photobiont strain. For generalists, a fungal system has been suggested which maintains the association with a particular strain for short-term advantages, allowing rapid exploitation and colonization, but which switches to new photobiont combinations that permit a fine-tuning of the symbiosis in order to persist in a changing environment and survive over a longer timescale (Nelsen & Gargas 2008). Thus, low selectivity would allow lichen-forming fungi to establish successful symbioses with locally-adapted photobionts in a wider range of habitats. However, the generalist pattern of *E. pedicellatum* presented in this study cannot hide the fact that this

species occupies a narrow ecological niche. Representatives of this species are predominantly found on coniferous trees in the very humid temperate and boreal Northern Hemisphere. Particularly in Atlantic Canada, they have been shown to be susceptible to air pollution (Cameron *et al.* 2013b) and the environmental effects of logging (Maass & Yetman 2002). In Alaska, *E. pedicellatum* is also found in a narrow ecological range, being largely confined to *Picea glauca* trees in the limited area of maritime boreal forests south of the Alaskan Range (Stehn *et al.* 2013).

Presence of the compatible *Rhizonema* strain in potential habitats in Newfoundland

This study found no significant difference between the presence of the *rbcLX* haplotype R1 in *F. asagrayana* and the occurrence of *C. palmicola* on the same tree in Newfoundland. Indeed, R1 incorporates the majority of the *Rhizonema* found living with the liverwort *F. asagrayana*, regardless of the presence of lichens on the tree. This result contradicts the expectation that the compatible photobiont of *E. pedicellatum* is more frequently found on trees where the core lichen *C. palmicola* occurs. This finding, while preliminary, supports the hypothesis that the *Coccocarpia-Erioderma* guild in Newfoundland is facilitated by local reservoirs of *Rhizonema* photobionts, either in association with *F. asagrayana* and/or free-living on the substratum, or both. Such a tripartite facilitation hypothesis has been suggested in greater detail in Cornejo & Scheidegger (2016), and we argue that cyanobacterial diversity in co-occurring symbiotic bryophytes needs to be included in metacommunity studies of lichens. However, more research on this topic needs to be undertaken before the interaction points between co-occurring bryophytes and lichens is more clearly understood. In the particular case of the compatible *Rhizonema* of *E. pedicellatum*, screening of the possible free-living *Rhizonema* on the substratum is

needed to better understand the role of *F. asagrayana* as photobiont facilitator.

Interestingly, there are *Rhizonema* strains of *E. pedicellatum* from trees in stands where neither *E. pedicellatum* nor *C. palmicola* was present. In addition, R1 integrates all samples of *C. palmicola* and *E. pedicellatum* from five different collection sites in Newfoundland, demonstrating that there is no geographical subdivision of specimens within this area. These results show that potential habitats of *E. pedicellatum* are colonized by compatible *Rhizonema*, living together with *F. asagrayana*, and demonstrate that the geographical distribution, and possibly also the ecological requirement of the photobiont of *E. pedicellatum* are wider than that of the lichen phenotype.

In conclusion, the photobiont selectivity of *E. pedicellatum* can be described as low globally, except for the high local selectivity in Newfoundland. Even though in Newfoundland *E. pedicellatum* was found to associate with R1-cyanobacteria, R3a-*Rhizonema* are also known to be compatible and present, being the most common symbiont of the co-occurring, sterile *Lichinodium* sp., and very closely related to the photobiont of *E. pedicellatum* from Norway. Additionally, even though it was found in one single specimen, the R3a-*Rhizonema* has here been shown to be capable of associating with the liverwort *F. asagrayana* on a tree without other lichens. Therefore, the exclusive association of *E. pedicellatum* with R1-*Rhizonema* in Newfoundland cannot be explained by the absence of R3a. Differences in photobiont selectivity varying between different regions have also been documented for green-algal lichens (Yahr *et al.* 2006; Muggia *et al.* 2013, 2014; Dal Grande *et al.* 2014) and cyanolichens (Otálora *et al.* 2010, 2013; Fedrowitz *et al.* 2012; O'Brien *et al.* 2013). It has been hypothesized that the symbiont diversity in these lichen species reflects one mechanism by which these symbioses respond to habitat variability and environmental change at a local scale (Rikkinen 2013). In this way, it may allow fungal hosts to associate with the cyanobacterial genotypes that are optimally adapted to the prevailing conditions.

In this context, our results provide further support for the geographical mosaic theory of co-evolution (Thompson 1999a, b, 2005) that states that adaptation varies through space and time in interactions between species, creating selection mosaics of co-evolving species above the level of local communities. However, further studies will need to be undertaken to test this hypothesis. A gap of knowledge in the historical relationship of populations of *E. pedicellatum*, for example, limits our understanding of spatial structure. Our data show that apparently Pacific populations are the primary source of *E. pedicellatum*, indicated by higher flexibility of the mycobiont to successfully establish the symbioses with several different *Rhizonema* genotypes. But recolonization after extinction by a subset of the metapopulation can also be the cause of a geographical mosaic (Gomulkiewicz *et al.* 2007). Another important source of uncertainty is our lack of knowledge on gene flow across landscapes and genetic drift among Atlantic and Pacific populations of *E. pedicellatum*, which could also have the potential to produce spatial variation in selectivity. Lastly, an occupancy survey of a large number of patches using neutral genetic markers is needed to estimate the importance of gene flow, random genetic drift and rate of population extinction and recolonization of *E. pedicellatum*.

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