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Research

Assessing population structure and host specialization in lichenized cyanobacteria

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Summary

- Coevolutionary theory predicts that the distribution of obligately symbiotic organisms will be determined by the dispersal ability and ecological range of both partners. We examined this prediction for lichen-forming fungi that form obligate symbioses with cyanobacteria.
- We compared genotypes of both partners of 250 lichens collected at multiple spatial scales in British Columbia, Canada. Multilocus sequence data collected from a subset of 128 of the specimens were used to determine the degree of recombination within the cyanobacterial populations.
- We found that six distinct clusters of cyanobacterial genotypes are distributed throughout the known global phylogeny of the genus *Nostoc*, and that each appears to be evolving clonally. Fungal specialization is high, with each species associating with either one or two of the cyanobacterial clusters, while cyanobacterial specialization varies, with clusters associating with between one and 12 different fungal species. Specialization also varies geographically, with some combinations restricted to a single site despite the availability of both partners elsewhere.
- Photobiont association patterns are determined by a combination of genetically based specificity, spatial population structure, and ecological factors and cannot be easily predicted by photobiont dispersal syndromes.

Introduction

Lichens are symbiotic associations between a fungus (mycobiont) and one or more photosynthetic partners (photobionts), usually green algae and/or cyanobacteria. This symbiotic association is generally thought to be obligate for the mycobiont (Honegger, 1996), although there do appear to be rare cases of species that are capable of facultative lichenization, a phenomenon that seems associated with loss of lichenization in lineages such as in the Stictidaceae (Lutzoni *et al.*, 2001; Wedin *et al.*, 2004). It has long been argued that *Trebouxia* and closely related *Asterochloris*, some of the most common lichen photobionts, are also obligately symbiotic (Ahmadjian, 1967). More recent observations have challenged this view (Bubrick *et al.*, 1984; Mukhtar *et al.*, 1994), but the ecological significance of free-living stages remains controversial. *Nostoc* and *Scytonema*, the most common cyanobacterial lichen photobionts, are known to occur free-living in a wide variety of habitats but it has been difficult to determine if the same species occur both free-living and symbiotically using traditional phenotypic classification. Recent molecular phylogenetic evidence has shown that free-living and lichenized *Scytonema* form evolutionary independent lineages (Lücking *et al.*, 2009), but *Nostoc* appears to be facultatively symbiotic (O'Brien *et al.*, 2005).

For most lichen-forming fungal species, reproduction is predominantly sexual, though a variety of asexual propagules are also produced by different species (Bowler & Rundel, 1975). Many of these asexual propagules are specialized structures that facilitate the codispersal of the mycobiont and photobiont (Büdel & Scheidegger, 1996), resulting in the vertical transmission of the photobiont from one generation to the next. While codispersal of sexually produced ascospores and photobiont cells has been documented for some species (Pyatt, 1973), ascospores are generally dispersed independently of the photobiont. The association must then be reconstituted with algae or cyanobacteria in the vicinity of the germinating ascospore, resulting in the horizontal transmission of the photobiont for each new generation. Given that about one-fifth of all known fungal species are lichenized (Kirk *et al.*, 2008), this presents a paradox: how can a horizontally transmitted obligate symbiosis give rise to such an evolutionarily successful and specialized group of organisms?

Several strategies have been proposed that might allow sexually reproducing lichen-forming fungi to reconstitute a lichen symbiosis in nature from one generation to the next. These include extracting photobiont cells from the thalli of other lichens (Friedl, 1987; Ott, 1987) or from the asexual propagules of other species (Rikkinen, 2003); and persistence by forming temporary associations with incompatible photobionts (Gaßmann & Ott,

2000) or in a free-living state (Etges & Ott, 2001) until a compatible photobiont is encountered. It has also been suggested that effective dispersal via ascospores is rare and that most dispersal is via asexual propagules or thallus fragments (Bowler & Rundel, 1975), an hypothesis that was recently confirmed for the lichen *Lobaria pulmonaria* (Dal Grande *et al.*, 2012). For lichens where sexual reproduction appears to be the predominant mode of dispersal, such as *Peltigera* (O'Brien *et al.*, 2009), selection on the mycobiont is likely to favor increases in the taxonomic range of compatible photobiont partners (reduced specificity; Smith & Douglas, 1987) in order to increase the probability that a compatible partner is encountered.

In the case of green algal lichens, most mycobiont species examined appear to be highly specific about which photobionts they lichenize with (Kroken & Taylor, 2000; Helms *et al.*, 2001; Piercey-Normore & DePriest, 2001; Hauck *et al.*, 2007). However, the degree of photobiont specificity and selectivity (preferential association with certain species/genotypes; Galun & Bubrick, 1984), collectively referred to as specialization (O'Brien *et al.*, 2005), can vary dramatically among closely related mycobiont species (Yahr *et al.*, 2004) and can also vary within a species across its range (Yahr *et al.*, 2006; Fernández-Mendoza *et al.*, 2011). Early molecular studies on cyanolichens reported that the mycobionts exhibited high photobiont specificity at the species level (Paulsrud *et al.*, 1998, 2000). More recent studies suggest that this may not be a general result (Wirtz *et al.*, 2003; O'Brien *et al.*, 2005; Elvebakk *et al.*, 2008), although it does appear to hold for some species (Myllys *et al.*, 2007; Otálora *et al.*, 2010; Fedrowitz *et al.*, 2011). At higher taxonomic levels, it has been shown that the same lineage of *Nostoc* participates in symbiotic associations with a wide range of lichen taxa, as well as plant groups including cycads, bryophytes, and the angiosperm *Gunnera* (O'Brien *et al.*, 2005), although epiphytic cyanolichens appear to be restricted to a lineage nested within *Nostoc* (Rikkinen *et al.*, 2002; Elvebakk *et al.*, 2008).

The contradictory results obtained for species-level photobiont specialization by mycobionts of cyanolichens may be partly a result of the failure to explicitly account for the local availability of different photobiont genotypes. The most definitive studies of photobiont specialization have compared the photobionts of each lichen species with the pool of photobiont genotypes represented by co-occurring species at the same locations (Yahr *et al.*, 2004; Fedrowitz *et al.*, 2011). By genotyping both partners, it is also possible to determine the role of vertical photobiont transmission in shaping population structure (Yahr *et al.*, 2004; Fernández-Mendoza *et al.*, 2011; Dal Grande *et al.*, 2012), which can mimic the patterns resulting from specialization. This study applies such an approach to study photobiont specialization in the lichen-forming fungal genera *Peltigera* and *Nephroma*. Most species in both genera form mostly bimembered lichens, where the mycobiont is associated with the cyanobacterium *Nostoc* (although see Henskens *et al.*, 2012). However, some species form trimembered lichens, where the green alga *Coccomyxa* is the main photobiont and *Nostoc* is an accessory photobiont which is restricted to specialized structures called cephalodia (Miadlikowska & Lutzoni, 2000, 2004). Samples were collected from five sites in British Columbia, Canada, separated by 15–150 km. Both fungal and cyanobacterial partners were genotyped using highly variable sequence markers. Three additional photobiont markers were sequenced from a subset of specimens in order to assess the extent of recombination. Photobiont sequences were compared with a global sampling of *Nostoc* sequences obtained from GenBank to determine which *Nostoc* lineages could act as photobionts for each mycobiont species (specificity). Photobiont sequences were also compared with the photobionts of all other species at the same site to quantify selectivity. Finally, photobiont genotypes were compared among mycobiont haplotypes within species to determine if patterns of association could be explained by vertical photobiont transmission within fungal clones.

Materials and Methods

All cyanolichen specimens were collected from moss-covered ground, rocks and tree trunks within 1-m-diameter circular plots separated by 5–60 m at five sites in British Columbia, Canada, as previously described (see Supporting Information, Fig. S1; O'Brien *et al.*, 2009). Since the internal transcribed spacer (ITS) is highly variable at the species level in *Peltigera* (O'Brien *et al.*, 2009), it was used to characterize mycobiont populations. Photobiont populations were characterized by sequencing the cyanobacterial gene *rbcX* and the intergenic spacer between *rbcL* and *rbcX* for each specimen (Rudi *et al.*, 1998). In addition to the specimens used by O'Brien *et al.* (2009), genomic DNA was isolated and fungal ITS sequences were obtained for 46 additional *Peltigera* specimens as well as 15 *Nephroma* specimens from the same sites, using the PUREGENE Ultrapure DNA extraction Kit (Qiagen) and the primers ITS1F and ITS4 (see Table 1). In total, our dataset included 250 ITS sequences representing 25 species (see Table S1). *rbcLX* was sequenced using a published reverse primer and a redesigned forward primer that annealed within the last 50 bp of the *rcbL* gene (Table 1). For a subset of specimens, we obtained sequences for three additional cyanobacterial genes: *nifV1* (121 specimens), *rpoC2* (116 specimens) and *trnL* (118 specimens) (see Table S1 for details). See O'Brien *et al.* (2009) for details about PCR and sequencing conditions. All sequences were assembled using Sequencher 4.2 (Gene Codes, Ann Arbor, MI, USA) and representatives of each unique sequence type were aligned using MacClade 4.0 (Maddison & Maddison, 2000). Sequences have been deposited in GenBank (accession numbers KC437590–KC437650 (ITS), KC437651–KC437917 (*rbcLX*), KC437918–KC438038 (*nifV*), KC438039–KC438154 (*rpoC2*), KC438155–KC438272 (*trnL*).

For each photobiont alignment, maximum-likelihood (ML) phylogenetic analyses were conducted using the HKY85 substitution model with gamma distributed rate variation (four categories), with a BioNJ starting tree and nearest neighbor interchange (NNI) branch swapping using PhyML (Guindon & Gascuel, 2003). Branch support was assessed using the approximate likelihood ratio test for branches (aLRT; Guindon *et al.*, 2010). The P6b loop of *trnL* sequences was considered for discrimination of allele types, but excluded from phylogenetic

Table 1 Primers for amplification and sequences of fungal (internal transcribed spacer (ITS)) and cyanobacterial markers

Locus	Primer name	Position[a]	Orientation	Primer sequence (all 5′ to 3′)	References
ITS	ITS1F	1733 (18S)	Forward	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
ITS	ITS4	60 (25S)	Reverse	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
rbcLX	cx	52 (*rbcS*)	Reverse	GGGGCAGGTAAGAAAGGGTTTCGTA	Rudi *et al.* (1998)
rbcLX	cz	1405 (*rbcL*)	Forward	GAGTTTGARGCAATGGATACC	This study
trnL	Leu1	6	Forward	TGTGGCGGAATGGTAGACGCTAC	Wright *et al.* (2001)
trnL	Leu2	72	Reverse	GACTTGAACCCACACGAC	Wright *et al.* (2001)
rpoC2	*rpoC1*_1852F	1852 (*rpoC1*)	Forward	GCBATTCAGGAAGCACTAGC	This study
rpoC2	*rpoC2*_455R	455 (*rpoC2*)	Reverse	CCTTGAGGATCTGCCATC	This study
nifV1	*nifV1*_289F	289	Forward	GTCTCTGGTATCCAMATYGC	This study
nifV1	*nifV1*_1044R	1044	Reverse	GCGCACTGCATCTAAAACAG	This study

[a]Positions correspond to coding sequences for *Nostoc* PCC 7120, except for ITS primers, which correspond to positions in *Saccharomyces cerevisiae* S288c.

analyses. The extent of interlocus recombination was characterized using Multilocus 1.2 (Agapow & Burt, 2001) as described in O'Brien *et al.* (2009).

Representatives of each unique *rbcLX* sequence type were aligned with published sequences from symbiotic *Nostoc* and free-living *Nostoc commune* (Rudi *et al.*, 1998; O'Brien *et al.*, 2005; Rajaniemi *et al.*, 2005; Stenroos *et al.*, 2006; Myllys *et al.*, 2007), with intergenic spacers excluded from the alignment. This dataset was analyzed with ML, using the general time-reversible (GTR) substitution model with gamma-distributed rate variation (four categories), and an estimated proportion of invariant sites using Garli (Zwickl, 2006), with default tree search settings. Branch support was assessed with 100 bootstrap replicates. The resulting phylogeny was rooted with *Anabaena* PCC7120.

Separate ITS alignments were made for each of the 25 mycobiont species, so that all nucleotide positions could be unambiguously aligned. Haplotype networks were inferred using statistical parsimony with multibase indels coded as single characters with TCS (Clement *et al.*, 2000).

Significance of associations between photobiont sequence types and mycobiont species and between photobiont clusters and sampling sites were assessed with Fisher's Exact Test using R (R Development Core Team, 2012), with redundant specimens from the same plot with identical mycobiont and photobiont sequences removed. Host specialization and spatial population structure were also quantified by grouping photobiont genotypes by mycobiont species or by sampling site and calculating linearized pairwise F_{ST} values. Only groups represented by 10 or more specimens were included in the comparisons. Significance was assessed using permutation tests. All population structure analyses were carried out using the program Arlequin 3.01 (Excoffier *et al.*, 2005).

Results

Photobiont phylogenies

All sequencing reactions produced clean reads with no evidence of secondary peaks, suggesting that a single photobiont genotype predominated in each lichen thallus. When analyzed with *rbcLX* sequences from a global sampling of *Nostoc* strains from a variety of symbiotic associations obtained from GenBank, photobiont strains from British Columbia (BC) comprised 30 distinct alleles distributed throughout the core lineage of *Nostoc* that includes most free-living and symbiotic isolates (clade II of Svenning *et al.*, 2005/*Nostoc* II of O'Brien *et al.*, 2005; Fig. 1a, see Fig. S2 for details). Twenty six of the 30 alleles formed six clusters of closely related alleles (labeled I–VI in the figures). One cluster of six alleles (IV) was made up exclusively of BC photobionts and a few rare alleles (13, 26) were on long branches, but the majority of BC photobiont sequences were very closely related to lichen photobionts from Europe and other locations. Ten BC photobiont alleles were identical to photobionts from lichens collected in Europe (1, 2, 3, 5, 7, 9, 12, 14, 15, and 17), including the four most common types (Fig. S2).

For the 128 specimens for which multiple photobiont loci were sequenced, 18 different alleles were recovered for *rbcLX*, 21 for *nifV1*, 12 for *rpoC2*, and 15 for *trnL* (Fig. 1). The same *rbcLX* allele was recovered from 80 of the 128 specimens (allele 2 in Fig. 1a). These specimens had three different *rpoC2* alleles, eight different *nifV1* alleles, and five different *trnL* alleles. The frequency of the most common allele was 55 for *rpoC2*, 54 for *trnL*, and 32 for *nifV1*. There was one other *rbcLX* allele (type 4) that comprised two or more alleles in all other phylogenies, while alleles 1 and 9 comprised two *nifV1* alleles each. There were also cases where strains with different *rbcLX* alleles had the same allele in another locus (alleles 1 and 11 in all three loci; alleles 2, 6 and 30 for *nifV1*; alleles 2 and 3 for *rpoC2*; alleles 2 and 6 and 25 and 27 for *trnL*). However, in all of these cases, strains with identical alleles at one locus had alleles that clustered together at all other loci and there were no cases where all four possible combinations of two alleles at each of two loci were present (the four gamete test; Hudson & Kaplan, 1985). Each of the six clusters identified in the *rbcLX* phylogeny were also monophyletic in the other gene trees. There was some incongruence between phylogenies at deeper nodes, including the phylogenetic positions of two singleton alleles (26 and 29), but the only topological conflict that was supported by bootstrapping involved the relationships among clusters II, III, and IV in *rbcLX* and *nifV1*. This congruence was reflected in the linkage disequilibrium calculations, as index of association values were extremely high ($I_A = 1.51$), even when the sample was clone-corrected ($I_A = 0.78$). These values were highly significant when compared with randomized datasets ($P < 0.001$).

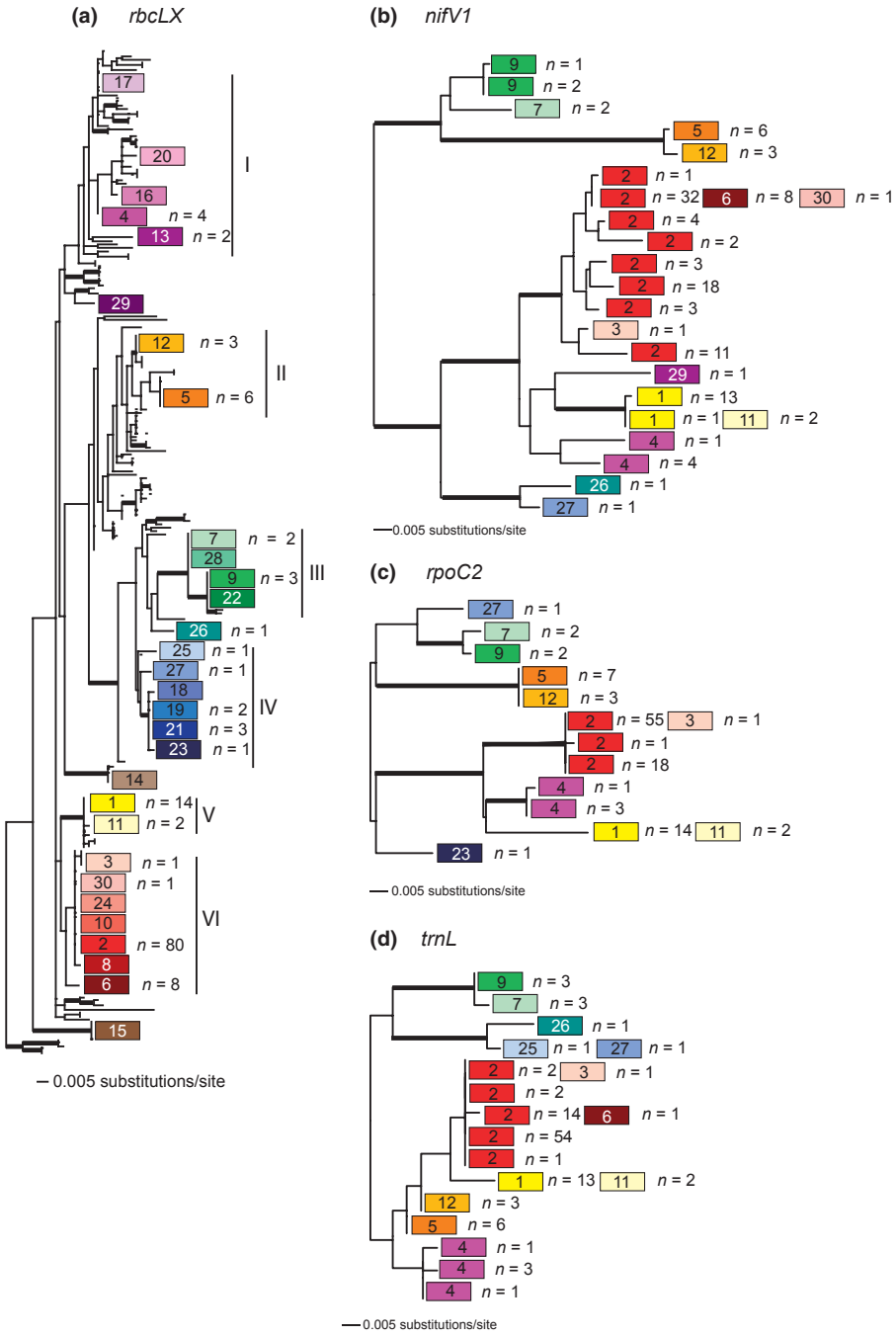

Fig. 1 Phylogenies for four photobiont loci: (a) *rbcLX* phylogeny. Colored boxes correspond to the *rbcLX* alleles recovered in this study (numbers are inside boxes). All other branches correspond to GenBank sequences from other localities (see Supporting Information Fig. S2). Roman numerals I–VI refer to photobiont sequence clusters referred to in the text. Numbers indicate the total number of photobionts with each given allele that were sequenced for the other genes (for a total of 128 specimens). For *nifV1* (b), *rpoC2* (c) and *trnL* (d) phylogenies, the distribution of *rbcLX* alleles is mapped on to the tree. The number of photobionts with a given combination of alleles for *rbcLX* and the gene used to infer the tree is given beside the boxes. Thickened branches indicate approximate likelihood ratio test $\geq$ 90%.

Mycobiont–photobiont comparisons

The most abundant mycobiont species present in the plots sampled was *Peltigera leucophlebia* (section *Chloropeltigera*), which is subdivided into three distinct clades following O'Brien *et al.* (2009; Fig. 2b). For the most abundant clade in this group, clade I, 44 mycobiont sequences were divided among eight different haplotypes (Fig. 2b). The two most common haplotypes comprised 33 of the 44 specimens, while the remaining six included four or fewer. The second most abundant group, clade III, was represented by 24 specimens divided among three fungal haplotypes, two of which were observed in a single specimen each, while clade II consisted of five specimens comprising three fungal haplotypes (Fig. 2b). Of these 72 lichen specimens, 58 had identical photobiont *rbcLX* sequences (allele 2) and six others had related photobionts in the same cluster (VI). Four additional photobiont alleles were recovered from the other eight specimens, which belonged to two other clusters (I and IV in Fig. 2a). Cluster I photobionts were found in both of the common *P. leucophlebia* clades while cluster IV photobionts were restricted to clade I.

The second most common mycobiont species in the sampling areas was *Peltigera aphthosa* (section *Peltidea*). Of 28 specimens examined, 23 had a single fungal haplotype, with the five other specimens each having a unique haplotype that differed from the

common haplotype by one or two mutations (Fig. 2b). Allele 2 *rbcLX* sequences were recovered from the photobionts of 13 specimens and a closely related photobiont allele was found in two others. The remaining 13 specimens had one of four related photobiont alleles from cluster IV. The sister species to *P. aphthosa*, *Peltigera britannica*, was only sampled 16 times, but one more fungal haplotype was recovered than for *P. aphthosa* (Fig. 2b). The fungal haplotypes were also more divergent, with a total tree length of 11 steps compared with six for *P. aphthosa*. Allele 2 photobionts were present in nine specimens and two related alleles were recovered from three others. A single cluster IV allele was recovered from the photobiont of three *P. britannica* specimens and a single specimen had a cluster I photobiont.

The third mycobiont species from section *Peltidea* present in the sample plots was *Peltigera malacea*. Unlike the other members of this section, which form trimembered thalli with cephalodia, *P. malacea* forms bimembered thalli with exclusively cyanobacterial photobionts. Three mycobiont haplotypes were recovered from 16 specimens for this species, with two of these haplotypes found in a single specimen each (Fig. 2b). Five photobiont *rbcLX* alleles were recovered from this species. Four of them clustered together (cluster III), while the fifth (allele 25), which was recovered from a single specimen, belonged to the related cluster IV (Fig. 2a).

For *Peltigera* section *Peltigera*, mycobiont species richness was much higher, with representatives of seven described species and three undescribed species present (Fig. 2b). In addition, *Peltigera ponojensis* was represented by two fungal haplotypes that differed by 12 mutations, suggesting the presence of an additional cryptic species. *Peltigera canina* and *Peltigera kristinssonii* were the most common species from this group, represented by 19 and 20 specimens respectively. Mycobiont haplotype diversity varied, from *Peltigera cinnamomea*, which was represented by five specimens, each with a different haplotype, to *P. kristinssonii* for which 17 of 20 specimens had identical haplotypes. Photobionts with *rbcLX* alleles belonging to cluster VI were present in all specimens of four species in this group (*Peltigera 'fuscopraetextata'*, *P. kristinssonii*, *P. ponojensis,* and *P.* sp. A), including the most common species (*P. kristinssonii*). All but one of the specimens of a fifth species (*Peltigera praetextata*) had cluster VI photobionts. Three other mycobiont species had photobionts with *rbcLX* sequences from two distinct clusters (V and VI for *P. canina* and *P. cinnamomea*; I and V for *P.* '*neocanina*') while *Peltigera membranacea* photobionts had *rbcLX* sequences from cluster V and two different unclustered alleles (14 and 15). Finally, *Peltigera degenii* was represented by a single specimen with allele 14 photobiont.

Six additional *Peltigera* mycobiont species from three sections were also collected, and two of these (*Peltigera neopolydactyla* and *Peltigera venosa*) also appeared to have additional cryptic mycobiont species based on ITS sequence divergence (Fig. 2b). These species were all uncommon, being represented by between two and five specimens and either one or two fungal haplotypes. Four of these species were associated with photobionts from a single *rbcLX* sequence cluster each, including all three species in *Peltigera* section *Horizontales* (*Peltigera collina*, *Peltigera horizontalis* and *Peltigera neckeri*), none of which associated with cluster VI photobionts, the most common photobiont cluster in 11 other *Peltigera* species. *P. venosa*, a trimembered species from *Peltigera* section *Phlebia*, was associated exclusively with cluster VI photobionts, while *Peltigera polydactylon* and *P. neopolydactyla* (*Peltigera* section *Polydactylon*), were both associated with photobionts with *rbcLX* sequences from cluster VI and one other cluster or unique allele.

For *Nephroma*, four mycobiont species were sampled. *Nephroma resupinatum* was represented by a single specimen, while *Nephroma bellum* and *Nephroma arcticum* were sampled twice each, and *Nephroma parile* was sampled 10 times (Fig. 2c). A single fungal ITS haplotype was recovered from each species except *N. bellum*, where each of the two specimens had a different haplotype. All 10 *N. parile* specimens had the same photobiont *rbcLX* allele, while *N. bellum* and *N. resupinatum* shared photobionts with the same allele. The photobiont *rbcLX* alleles from these three species clustered together (cluster II) and were not found in any of the *Peltigera* species. The two specimens of *N. arcticum*, a trimembered species, had photobionts with different *rbcLX* sequences that did not group with those of the other *Nephroma* species. One was part of cluster IV, while the other (allele 26) was intermediate between clusters III and IV.

Geographic patterns

A large majority of specimens were collected from the two sites near Clearwater BC (southern sites), with 169 specimens collected from the Spahats Creek site and 56 from the Battle Creek site compared with between six and 19 specimens from the three northern sites in the Cariboo mountains (Barkersville, Cameron Ridge and Ghost Lake; Fig. S1). There were large mycobiont species composition differences between the sites, with nine species collected exclusively from the southern sites and five collected only in the northern sites (Fig. 2). Of the 12 species that were collected at both southern and northern sites, seven had identical mycobiont ITS haplotypes recovered from both. One to three mutations separated northern and southern haplotypes for all other mycobiont species, except for *P. venosa* where haplotypes were separated by 12 mutations and likely represent different species. Eight mycobiont species had identical photobiont *rbcLX* alleles in southern and northern sites while two of the others had photobionts from the same sequence clusters. Two of the species were associated with different photobiont clusters in southern and northern sites; however, *P. britannica* was associated exclusively with cluster VI photobionts in the southern sites and with photobionts with alleles 4 (cluster I) and 19 (cluster IV) at the northern sites. Likewise, *P. membranacea* was associated with allele 1 (cluster V) photobionts at the southern sites and photobionts with *rbcLX* alleles 14 and 15 at the northern sites.

Photobiont population structure

When photobionts from different lichen species were compared using F_{ST}, the photobionts of *N. parile* and *P. malacea* were highly structured in comparison to all other species ($F_{ST} = 0.59–1.00$;

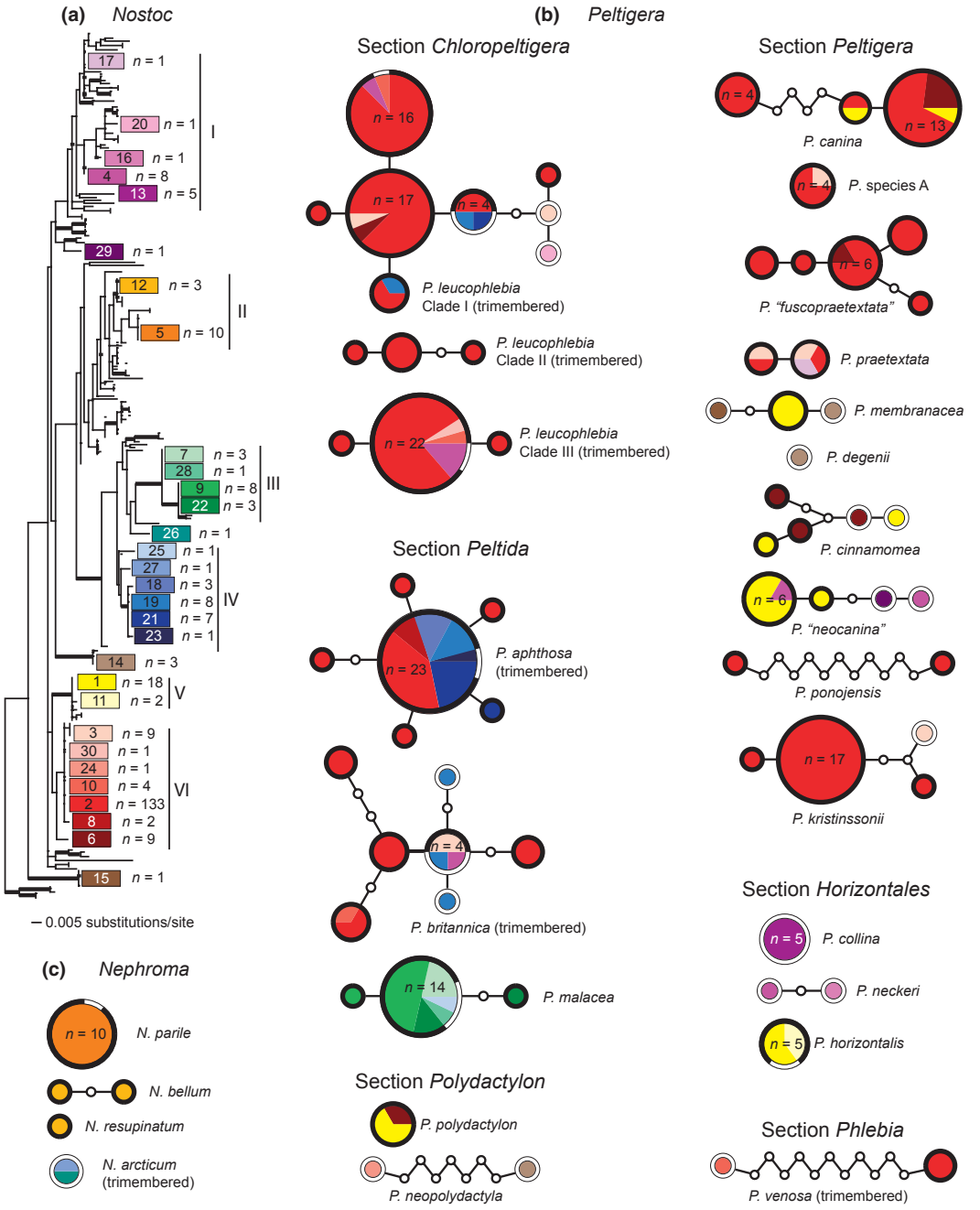

Fig. 2 Phylogenetic relationships of photobiont *rbcLX* alleles (a) associated with each mycobiont haplotype recovered for species of *Peltigera* (b) and *Nephroma* (c), for a total of 250 specimens. *Peltigera* sections are as defined by Miadlikowska & Lutzoni (2000). Sizes of circles are proportional to the number of mycobionts with each haplotype. Small empty circles indicate unsampled intermediate haplotypes. Color-coding indicates the proportion of specimens containing the different photobiont alleles shown in (a). Black borders around circles indicate that specimens were collected at one of the southern sites (Spahats Creek or Battle Creek). White borders indicate that specimens were collected at one of the northern sites (Barkersville, Cameron Ridge or Ghost Lake). All taxa with *Coccomyxa* as their main photobiont and with *Nostoc* as an accessory photobiont (i.e. within cephalodia) are labeled as trimembered (one mycobiont + two photobionts) in (b) and (c). All other lichens are bimembered (one mycobiont + one photobiont) with *Nostoc* as their main photobiont (i.e. without cephalodia). See Supporting Information Fig. S2 for more information about the *Nostoc* phylogeny.

Table 2). These values were highly significant. *P. aphthosa* photobionts were also significantly different from those of all other species, except for the closely related species *P. britannica* ($F_{ST} = 0.10$ for *P. britannica*, $F_{ST} = 0.30$–0.61 for other species). F_{ST} values for all other comparisons ranged from −0.01 to 0.16. Some of these values were significant, but none of the other species with $n \geq 10$ had photobionts that were significantly different from all other species. Pairwise F_{ST} values were significant for all comparisons between sites, but values were much lower for the comparison between Spahats Creek and Battle Creek (both southern sites; $F_{ST} = 0.04$) than for any of the other comparisons ($F_{ST} = 0.15$–0.42; Table 3).

Table 2 Pairwise F_{ST} values for photobionts, structured by mycobiont species (only species with $\geq$ 10 specimens included)

	N. parile	*P. aphth.*	*P. brit.*	*P. can.*	*P. leu.* I	*P. 'fusco.'*	*P. krist.*	*P. leu.* III
N. parile ($n = 10$)								
P. aphthosa ($n = 28$)	**0.61**							
P. britannica ($n = 16$)	**0.73**	0.10						
P. canina ($n = 18$)	**0.89**	**0.34**	**0.12**					
P. leucophlebia Clade I ($n = 44$)	**0.81**	**0.30**	0.02	0.04				
P. 'fuscopraetextata' ($n = 13$)	**0.96**	**0.33**	0.09	0.01	−0.01			
P. kristinssonii ($n = 20$)	**1.00**	**0.39**	**0.16**	0.15	0.01	0.02		
P. leucophlebia Clade III ($n = 24$)	**0.92**	**0.36**	0.10	0.08	0.01	0.02	0.07	
P. malacea ($n = 16$)	**0.94**	**0.59**	**0.75**	**0.87**	**0.84**	**0.92**	**0.95**	**0.90**

Values in bold indicate significant ($P \leq 0.05$) values as assessed by Bonferroni corrected permutation tests.

Table 3 Pairwise F_{ST} values for photobionts, structured by site (Cameron Ridge excluded)

	Spahats Creek	Battle Creek	Ghost Lake
Spahats Creek ($n = 168$)			
Battle Creek ($n = 56$)	0.04		
Ghost Lake ($n = 22$)	**0.30**	**0.42**	
Barkersville ($n = 14$)	**0.15**	**0.19**	**0.24**

Values in bold indicate significant ($P \leq 0.05$) values as assessed by Bonferroni corrected permutation tests.

Discussion

Multilocus sequence data from the photobionts of the lichens collected for this study strongly indicate that the population structure of symbiotic *Nostoc* is clonal. All multilocus genotypes observed were compatible with clonal descent, and linkage disequilibrium as measured by the Index of Association is high (0.78 after clone correction). The phylogenies from the different loci are also highly congruent, as the only topological conflict that received significant bootstrap support involved the relationships among clusters II, III, and IV in *rbcLX* and *nifV1*. The finding of a clonal population structure for *Nostoc* contradicts reports of incongruence between loci from other studies (Rudi *et al.*, 1998; O'Brien *et al.*, 2005; Kaasalainen *et al.*, 2012). It may be that genetic exchange is so rare that no recombinants were present in our sample, or the frequency of recombination may vary geographically or be higher in free-living populations.

The lichen photobionts sampled from British Columbia represent a large fraction of the worldwide phylogenetic diversity of *Nostoc* (Figs 1a, S2), but the number of different sequence types present at each site was quite low, with a maximum of 17 sequence types at the most extensively sampled site. There was some evidence of isolation by distance, as there was much less population differentiation between the Spahats Creek and Battle Creek sites, which are located within 15 km of each other, than between any other pair of sites (Table 2). These results contradict the traditional view that microbes are not dispersal-limited (Beijerinck, 1913; Baas-Becking, 1934) but are similar to what has been observed for other groups of cyanobacteria (Papke *et al.*, 2003; Gugger *et al.*, 2005).

For many of the *Peltigera* species sampled, the most common photobiont *rbcLX* allele type was the same (allele 2). Most of these mycobiont species also associate with some of the closely related photobiont alleles from cluster VI. However, these photobiont types were completely absent from seven of the *Peltigera* species sampled. Many of these species were sampled at low frequency, and in some cases they were sampled only from the northern sites where photobiont type 2 was less common. Nevertheless, given the high frequency of *rbcLX* allele 2 in the species where it does occur, its absence from these seven species is highly significant ($P < 10^{-5}$). Similarly, the second most common photobiont type (*rbcLX* allele 1), which was the predominant photobiont type for four *Peltigera* species, did not occur in any of the species that use allele 2 photobionts, except *P. canina* (Fig. 2b).

A third case of a cluster of photobionts being restricted to a subset of species is cluster IV, which was only recovered from *N. arcticum*, *P. aphthosa*, *P. britannica*, *P. malacea* and one of the three clades of *P. leucophlebia* that were shown to be reproductively isolated from each other by O'Brien *et al.* (2009). These photobiont *rbcLX* alleles are found at high frequency in specimens collected from the northern sites; in the southern sites, they were found only in *P. aphthosa* and in one specimen of *P. leucophlebia* clade I. These differences in photobiont association between the two regions are highly significant ($P = 0.0004$ when all trimembered species are pooled). Such changes in patterns of host association with geography have also been documented for green algal lichens (Yahr *et al.*, 2006) and are a strong prediction of the geographic mosaic theory of coevolution (Thompson 2005). It is interesting to note that this third group of photobiont types (cluster IV) is only found in trimembered lichens (except for one *P. malacea* specimen, discussed later), meaning that these *Nostoc* types were found almost exclusively as accessory photobionts isolated in cephalodia.

In addition to the photobiont specialization outlined earlier, there were also several cases of high reciprocal specificity, where all specimens of a lichen species (or of two related species) contained a single photobiont type or a single cluster of photobiont types that were not recovered from any other lichen (*P. malacea*, *N. parile* and *N. bellum*/*N. resupinatum*). Furthermore, all photobiont *rbcLX* sequences from specimens representing these three species collected in locations other than British Columbia,

including eastern North America, Asia and Europe are identical to the sequences obtained here, or cluster with them to the exclusion of all other sequence types (see Fig. S2). In the case of the *Nephroma* spp., however, Myllys *et al.* (2007) found *Lobaria pulmonaria* photobionts with identical *rbcLX* sequences to both *Nephroma* photobiont types, and *Parmeliella triptophylla* photobionts that were identical to the *N. bellum/N. resupinatum* photobiont type. Neither of these species occurred in our study sites. This suggests that a similar pattern of low but variable specialization by the photobionts and higher specialization by the mycobionts is occurring in *Peltigera* and *Nephroma*, but that the different *Nephroma* photobiont lineages are more closely related to each other.

In contrast to *Nephroma*, 15 of 16 *P. malacea* photobionts recovered in this study grouped together with nine of 10 photobionts from *P. malacea* from eastern North America, Europe and Asia, forming a well-supported cluster to the exclusion of all other *Nostoc* sequences, while the remaining two sequences of the *Nostoc* found in *P. malacea* (one from Europe and British Columbia type 25), fall within a well supported group including the *P. malacea*-specific cluster and cluster IV (see Fig. S2). *P. malacea* is therefore an example of extreme one-to-one reciprocal specificity, a phenomenon observed in five different species of *Collema* and *Leptogium* (Otálora *et al.*, 2010). In the case of *Collema*/*Leptogium*, high reciprocal specificity was restricted to species with specialized codispersal structures (isidia), while *P. malacea* does not produce isidia or soredia. *P. malacea* also rarely produces ascospores and reproduction appears to be primarily clonal, probably through thallus fragmentation (O'Brien *et al.*, 2009). However, it seems unlikely that codispersal of the partners via thallus fragments could generate the pattern observed here, because of the diversity of different sequence types for both partners. It is more likely that some form of genetically determined specificity is operating.

The cluster containing *P. malacea* photobionts forms a strongly supported monophyletic group with cluster IV, which is restricted to trimembered *Peltigera* and *Nephroma* lichens, as well as photobionts of two *Sticta* species (Fig. S2). *P. malacea* is closely related to these trimembered *Peltigera* lichens (Miadlikowska & Lutzoni, 2004) and is very similar in morphology, chemistry and ecology (Vitikainen, 1994). These trimembered species can form bimembered cyanomorphs under the appropriate environmental conditions (Brodo & Richardson, 1978; Stocker-Wörgötter & Türk, 1994; Stocker-Wörgötter, 1995), suggesting that *P. malacea* may have evolved from the cyanomorph of a trimembered species. If the common ancestor of photobiont clusters III and IV evolved high specificity for trimembered *Peltigera* spp., possibly accompanied by reduced motility restricting these *Nostoc* strains to cephalodia (Paulsrud *et al.*, 2001), they may have cospeciated along with the mycobiont.

When photobiont association patterns are compared with the phylogenetic relationships of the mycobionts (Miadlikowska & Lutzoni, 2000; Miadlikowska *et al.*, 2003; O'Brien *et al.*, 2009), there are some examples of phylogenetic conservatism, where sister mycobiont species associate with the same photobiont types (photobiont *rbcLX* allele 14 found only in *P. degenii* and *P. membranacea*; allele 1 found in *P. cinnamomea* and *P.* '*neocanina*'). However, most patterns of photobiont specialization did not reflect the phylogenetic relationships of the mycobionts, suggesting that it must be either an evolutionarily plastic trait or driven by ecological differences.

It has been suggested that epiphytic and terricolous lichens use different lineages of photobiont (Rikkinen *et al.*, 2002; Rikkinen, 2003; Elvebakk *et al.*, 2008), but this seems unlikely in this case because different lichen-forming fungal species were collected from the same plot and ecological niche, that is, associated with mosses growing on soil and rocks. Ecological conditions were also unable to explain the patterns of photobiont specificity observed by Myllys *et al.* (2007) and Stenroos *et al.* (2006). Most lichenized *Nostoc* strains studied by Rikkinen *et al.* (2002) fell into two clades, with one comprising photobionts of epiphytic lichens (*Nephroma*, *Parmeliella*, *Lobaria*) and the other comprising those of terricolous lichens (primarily *Peltigera* and two terricolous species of *Nephroma*). However, the latter clade had low phylogenetic support in their analysis and has not been recovered as monophyletic by others (O'Brien *et al.*, 2005; Stenroos *et al.*, 2006; Elvebakk *et al.*, 2008). The '*Nephroma* guild' lineage is recovered as monophyletic in our analysis (see clade B in Fig. S2), but *Peltigera* photobionts form at least six distinct lineages. The photobionts of *N. arcticum* (a terricolous *Nephroma* species forming a trimembered association; *Nostoc rbcLX* alleles 26 and 27) did not group with the other *Nephroma* photobionts, but they were also distinct from *Peltigera* photobionts. However, both *N. arcticum* specimens were collected at the Ghost Lake site, where sampling was not as extensive as for some of the other sites, so it is possible that the *N. arcticum* photobiont types may be present in some of the *Peltigera* spp. at that site.

All of the mycobiont species that we sampled form associations with a very restricted subset of the locally available photobiont genotypes. This high degree of selectivity in a horizontally transmitted obligate symbiosis suggests that availability of suitable cyanobacterial partners is not limiting, at least at the locations we sampled. This is likely a result, at least in part, of the fact that many mycobiont species can share the same photobiont genotypes, that is, photobionts being generalists, allowing facilitation through photobiont sharing (Rikkinen, 2003). Despite the overall high degree of specialization demonstrated by the mycobiont, most well-sampled *Peltigera* spp. do associate with at least two clusters of *Nostoc* sequence types. This flexibility may increase the probability that ascospores are dispersed to areas where compatible photobiont partners are present or may allow ecological specialization if different photobiont lineages are adapted to different microclimatic conditions. This may help to explain the extremely wide geographical range and habitat diversity of many species of *Peltigera* (Martínez *et al.*, 2003).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sampling sites for this study.

Fig. S2 Phylogenetic placement of photobiont alleles within *Nostoc* s. str.

Table S1 Mycobiont and photobiont genotypes for each specimen included in this study, with GenBank accession numbers

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