Cyanobacteria and their toxins in lichen symbiosis

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Academic dissertation

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Contents

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I JR had the idea and UK, JR, JJ and DF designed the study. Lichen specimens were collected by UK and JR. LC-MS analyses were performed by UK and JJ, other labwork by UK and DF, and phylogenetic analyses by DF. UK wrote the paper as main author together with JR, DF, JJ, and KS.

II UK and JR designed the study and collected most of the lichen specimens. UK, MW and JJ performed the LC-MS analyses and UK other labwork and phylogenetic analyses. UK wrote the paper as main author together with JR and DF. All authors commented.

III KF developed the study together with JR and UK. Specimens were collected by several people. KF conducted the labwork together with UK. UK performed phylogenetic analyses and KF constructed the haplotype net. KF wrote the paper as main author together with JR and UK.

IV UK designed the study, conducted the labwork, performed the analyses with JR and wrote the paper as main author together with JR. All authors commented.

Abbreviations

Adda	(*2S,3S,8S,9S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ADMAdda	*O*-acetyl-*O*-demethylAdda
Ala	alanine
Arg	arginine
DMAdda	demethylAdda
Glu	glutamic acid
ITS	internal transcribed spacer
LC	liquid chromatography
Leu	leucine
MC	microcystin
mcyE	microcystin synthetase gene E
Mdha	*N*-methyldehydroalanine
Mdhb	2-(methylamino)-2-dehydrobutyric acid
MeAsp	erythro-ß-methylaspartic acid
MS	mass spectrometry
Nod	nodularin
PCR	polymerase chain reaction
TLC	thin layer chromatography
trnL	$tRNA^{Leu}$ (UAA) intron

Abstract

Lichens are symbiotic associations between a fungus (mycobiont) and a photosynthetic partner (photobiont) which may be a green alga or cyanobacterium (cyanobiont). In lichen symbiosis the mycobiont lives on sugars photosynthesized by the photobiont and, in cyanobacterial symbiosis, also nitrogen compounds are provided to the fungal host. Several cyanobacterial genera are known to associate with lichen forming fungi but by far the most common cyanobacterial genus in lichen symbioses is *Nostoc*. Lichen-symbiotic *Nostoc* is a diverse group including at least two distinct phylogenetic lineages which tend to associate with different groups of lichen mycobionts.

Microcystins and nodularins are small, cyclic, hepatotoxic peptides responsible for poisonings of humans and animals. They are produced by aquatic, bloom forming cyanobacteria of several different genera and found in fresh and brackish waters around the world. The previously known microcystin producers of the genus *Nostoc* include the lichen associated cyanobacterium *Nostoc* sp. IO-102-I isolated from Finland, and some aquatic strains from Brazil, Finland, and India. While all producers of nodularin were previously thought to belong to the genus *Nodularia*, it has recently been shown that also some *Nostoc* strains isolated from cycad roots can produce nodularin.

The aim of this study was to find out which cyanobacterial toxins are produced in lichen symbiosis and how widespread this production is, both from the geographical and lichen-symbiotic perspective. In addition I wanted to broaden the knowledge on lichen-symbiotic cyanobacteria and symbiont selectivity in lichen symbiosis. The study was based on the analysis of over 800 cyanolichen specimens collected from different parts of the world, mainly analysed with molecular biological methods and liquid chromatography-mass spectrometry.

The results show that hepatotoxic microcystins are produced *in situ* in lichen symbioses by symbiotic cyanobacteria, and that these compounds are produced quite commonly in many different lichen genera all around the world. Also nodularin is produced in some lichens. The cyanobacterial toxins may act as grazing deterrents and provide some protection to the thallus. However the actual consequences to grazers and the faith of the toxins in the food chain remain unknown.

The chemical and genetic diversity of microcystin production in lichens was remarkable. The evolution of this diversity may be related to genetic bottlenecks that commonly occur during the lifecycle of symbiotically dispersing cyanobacteria and the concurrent close association with the fungal hosts. The presently known distribution of toxin-producing cyanobacteria in lichens was found to concentrate into certain taxonomic groups within the Lobariaceae, Nephromataceae, and Peltigeraceae (Peltigerales, Ascomycota). The diversity of microcystin structures correlated with the genetic identity of *Nostoc* symbionts in different lichens, but also geographical patterns seemed to exist.

Symbiont selection in the lichen genus *Nephroma* was found to be more specific locally than globally, and the identity of the cyanobiont to differ between bi- and tripartite members of the genus.

Summary

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1. Introduction

Cyanolichens

Lichens are symbiotic associations between a fungus (mycobiont) and a photosynthetic partner (photobiont) which may be a green alga or cyanobacterium (cyanobiont). It is estimated that approximately 13% of all lichen species have cyanobacterial symbionts as a primary photobiont (bipartite lichens) or secondary photobiont (tripartite lichens) (Friedl & Büdel 1996; Honegger 1996; Rikkinen 2002). Tripartite lichens have green algae as the primary photobiont and the cyanobacterial symbionts are usually located in specified structures called cephalodia. In lichen symbiosis the mycobiont lives on sugars photosynthesized by the photobiont. In case of symbiotic cyanobacteria also nitrogen compounds are available: the cyanobiont fixes atmospheric nitrogen into ammonia, nitrates or nitrites to be absorbed by the fungal host (Friedl & Büdel 1996).

Cyanolichens are not a monophyletic group but instead belong to several orders within the Ascomycota, and especially to the different families within the order Peltigerales (Fig. 1). *Nephroma* and *Peltigera* are genera of mostly bipartite foliose cyanolichens with nearly a cosmopolitan distribution (James & White 1987; White & James 1988; Holtan-Hartwig 1993; Vitikainen 1994a, b; Miadlikowska & Lutzoni 2000; Lohtander *et al.* 2002; Martinez *et al.* 2003; Vitikainen 2007 a, b; Serusiaux *et al.* 2011). *Peltigera* is notorious for its taxonomic challenges and known to include several difficult species complexes (Goffinet *et al.* 2003; Miadlikowska *et al.* 2003; Serusiaux *et al.* 2009).

Lichen-symbiotic cyanobacteria

Representatives from several cyanobacterial genera, e.g. *Chroococcidiopsis*, *Gloeocapsa*, *Scytonema*, and *Stigonema*, are known to associate with different lichens but by far the most common cyanobacterial genus in lichen symbioses is *Nostoc* (Rikkinen 2002). The genus *Nostoc* includes filamentous, non-branching cyanobacteria that produce heterocysts, cells specialized in nitrogen fixation, and hormogonia, which are motile filaments often involved in initiating the symbiotic associations (Adams & Duggan 2012). In addition to lichens *Nostoc* forms several other symbiotic associations (Papaefthimiou *et al.* 2008): with thalloid bryophytes (Adams & Duggan 2008; Rikkinen & Virtanen 2008), cycads (Costa *et al.* 2004; Gehringer *et al.* 2010; Yamada *et al.* 2012; Thajuddin *et al.* 2010), the angiosperm *Gunnera* (Nilsson *et al.* 2000; Svenning *et*

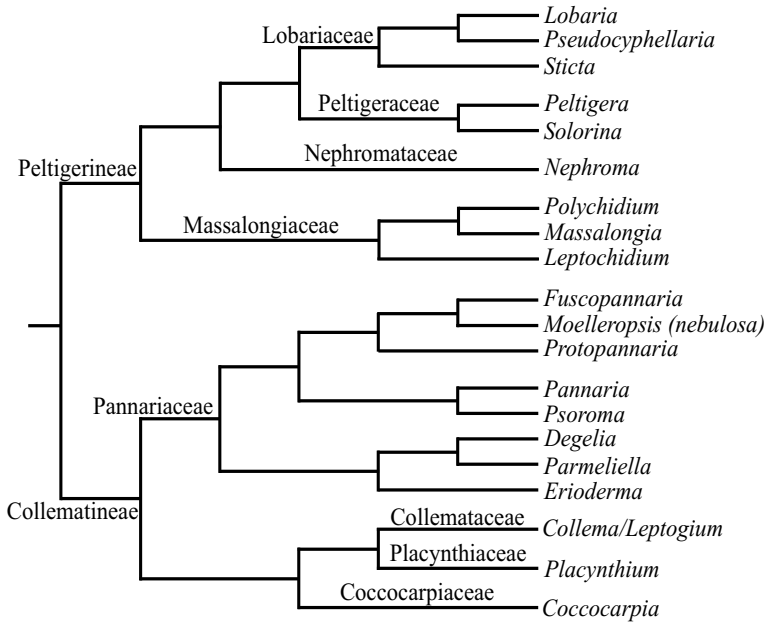

Figure 1. Outline of phylogenetic relationships within the Peltigerales according to Ekman & Jorgensen (2002), Hibbett *et al*. (2007), Schoch *et al*. (2009), Wedin *et al*. (2009), and Schmull *et al*. (2011).

al. 2005) and the glomeromycete *Geosiphon* (Gehrig *et al*. 1996; Schüßler & Wolf 2005).

Nostoc is the primary cyanobiont within lichen species of the Peltigerales (Rikkinen 2002; Lücking *et al*. 2009). Several studies have shown that generally most lichen mycobionts tend to associate with a restricted number of different *Nostoc* genotypes or genotype groups (e.g. Oksanen *et al*. 2002; Rikkinen *et al*. 2002; O'Brien *et al*. 2005; Otálora *et al*. 2010; Fedrowitz *et al*. in revision), with a few exceptions in some lichen species and certain environments (Wirtz *et al*. 2003; Kaasalainen *et al*. 2009). Lichen-symbiotic *Nostoc* represent a complex group including two distinct phylogenetic lineages which tend to associate with different groups of lichen species, both of these having the capacity to form functional guilds (Rikkinen *et al*. 2002; Rikkinen 2003, 2004; O'Brien *et al*. 2005; Myllys *et al*. 2007). One of these cyanobacterial lineages is well defined and, as far as is presently known, found almost exclusively in symbiosis with lichen forming fungi, mainly in association with species of *Nephroma, Leptogium, Lobaria*, *Pannaria*, *Parmeliella*, *Pseudocyphellaria*, and *Sticta* (Rikkinen *et al*. 2002; Summerfield & Eaton-Rye 2006; Myllys *et al*. 2007; Elvebakk *et al*. 2008; Otálora *et al*. 2010; Fedrowitz *et al*. 2011; Olsson *et al*. 2012). The second cyanobacterial lineage is much more diverse and includes, in addition to lichen cyanobionts, also plant cyanobionts and free-living *Nostoc* (Rikkinen *et al*. 2002; Lohtander *et al*. 2003; O'Brien *et al*. 2005; Myllys *et al*. 2007; Rikkinen & Virtanen 2008). These *Nostoc* genotypes associate with many different lichen species, but especially with ones belonging to the genus *Peltigera* (Paulsrud & Lindblad 1998; Paulsrud *et al*. 1998, 2000, 2001; Rikkinen *et al*. 2002; O'Brien *et al*. 2005; Myllys *et al*. 2007).

Cyanobacterial toxins microcystin and nodularin

Microcystins and nodularins are small cy-

clic peptides implicated in poisonings of humans and animals (Sivonen 2009). They are associated with bloom forming cyanobacteria in many fresh and brackish water bodies around the world. Microcystins are produced by many different cyanobacterial genera and for example genera *Anabaena*, *Hapalosiphon*, *Microcystis*, *Nostoc,* and *Planktothrix* contain microcystin-producing strains (Sivonen 2009). The toxicity of different cyanobacterial strains varies greatly and, typically, even within a single cyanobacterial species, some but not all strains produce microcystins (Sivonen & Jones 1999). The previously described microcystin producers of the genus *Nostoc* include the lichen-associated strain *Nostoc* sp. IO-102-I isolated from Finland (Oksanen *et al.* 2004a), and some aquatic *Nostoc* strains from Brazil, Finland, and India (Sivonen *et al.* 1990; Bajpai *et al.* 2009; Genuário *et al.* 2010). The only producers of nodularin were long thought to belong to the genus *Nodularia*, but very recently it was shown that also some *Nostoc* strains isolated from cycad roots produced nodularin (Gehringer *et al.* 2012).

Structure

Microcystins have a common chemical structure of cyclo(*D*-Ala^1–X^2–*D*-MeAsp^3–Z^4–Adda^5–*D*-Glu^6–Mdha^7), where X and Z are variable *L*-amino acids, *D*-MeAsp is *D*-erythro-ß-methylaspartic acid, Adda (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is *N*-methyldehydroalanine (Fig. 2; Sivonen 2009). Microcystin contains a number of nonproteinogenic amino acids (Sivonen & Börner 2008), and there are over 100 published structures varying in the type of amino acids incorporated into the peptide, demethylation of MeAsp and Mdha, and modification to the Adda side chain (Neffling 2010). Typically one cyanobacterial strain can produce several variants simultaneously even though only one or two of the variants are abundant (Sivonen 2009).

The structure of nodularin is quite similar to that of microcystin, but the amino acids in positions one and two are missing (Fig. 2). The resulting pentapeptide structure cyclo(D-MeAsp^1–L-Arg^2–Adda^3–D-Glu^4–Mdhb^5), where Mdhb is 2-(methylamino)-2-dehydro-

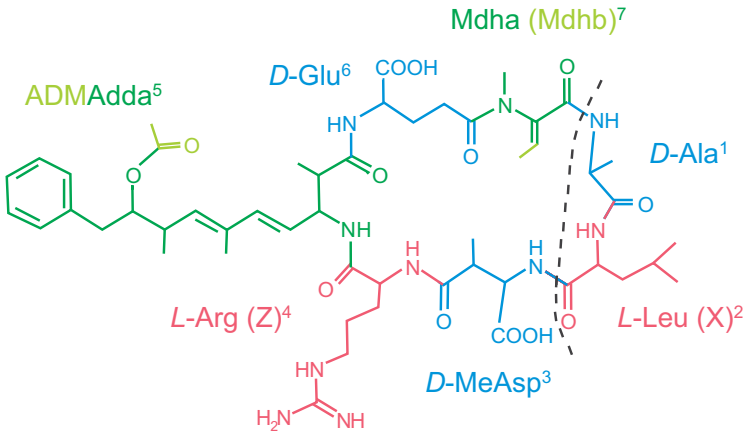

Figure 2. Structure of microcystin and nodularin. In nodularin *D*-Ala^1 and *L*-Leu^2 are missing (dash line) and the seventh amino acid (usually Mdha in microcystin) is replaced with Mdhb (difference in structure indicated with lighter green). The peculiar amino acid Adda is in lichen-associated microcystins often replaced with ADMAdda (*O*-acetyl-*O*-demethylAdda; difference in structure indicated with lighter green).

butyric acid, seem to be less variable, and only a few different nodularin variants have been found in nature (Sivonen 2009).

Toxicity and function

Microcystins and nodularins are potent inhibitors of eukaryotic protein phosphatases 1 and 2A and highly toxic: the intra-peritoneal mouse toxicities (LD_{50}) vary in the range 50–300 μg kg^{-1} body weight (MacKintosh *et al.* 1990; Honkanen *et al.* 1991; Sivonen & Jones 1999). The toxicity values follow the toxin structure, the most lethal (to mice) being microcystin MC-LR and Nod-R (Sivonen & Jones 1999). The use of microcystin-contaminated water in renal dialysis is held responsible for the deaths of 60 patients in Brazil (Jochimsen *et al.* 1998), and the compound is also suspected to act as a tumor promoter (Nishiwaki-Matsushima *et al.* 1992). Nodularin is also carcinogenic (Ohta *et al.* 1994). Most microcystins and nodularins are hydrophilic compounds and therefore unable to directly penetrate the lipid membranes of the cell. These toxins are uptaken into cells through membrane transporters, which in mammals restricts the damage mainly in the liver (Eriksson *et al.* 1990; Meriluoto *et al.* 1990).

Since the common ancestor of microcystin-producing cyanobacteria is thought to predate the eukaryotic lineage (Rantala *et al.* 2004) the compound did not, at least originally, evolve against grazing, and the actual function of these peptides has inspired several theories. Microcystin has been proposed to protect the cell against stress caused by iron or reactive oxygen species (Alexova *et al.* 2011), or to be involved in cellular interactions and/or colony formation (Schatz *et al.* 2007; Zilliqes *et al.* 2008).

Biosynthesis and evolution

Microcystins and nodularins are synthesized on large, mixed nonribosomal and polyketide synthetases in a programmed biosynthetic event (Nishizawa *et al.* 1999, 2000; Tillett *et al.* 2000; Moffitt & Neilan 2001; Christiansen *et al.* 2003; Rouhiainen *et al.* 2004). The microcystin gene cluster spans approximately 55 kb (Nishizawa *et al.* 2000; Tillett *et al.* 2000; Christiansen *et al.* 2003; Rouhiainen *et al.* 2004), and the sporadic distribution of microcystin and nodularin production among cyanobacteria is thought to be explained by multiple losses of the gene cluster, even though also horizontal gene transfer has been discussed as a possible mechanism (Otsuka *et al.* 1999; Tillett *et al.* 2001; Mikalsen *et al.* 2003; Rantala *et al.* 2004; Jungblutt & Neilan 2006; Christiansen *et al.* 2008). Nodularin synthetase genes are thought to be derived from the microcystin gene cluster by a recent deletion and mutation, where nodularin synthetase cluster lost the modules corresponding to parts of the *McyA* and *McyB*, and the remnants were fused together to form *NdaA* (Moffitt & Neilan 2004; Rantala *et al.* 2004).

2. Aim of the thesis

The aim of this thesis was to find out which cyanobacterial toxins are produced in lichen symbiosis and how widespread this phenomenon is, both from geographical and lichen-symbiotic point of view (Chapters I, II and IV). In addition I wanted to increase knowledge about lichen-symbiotic cyanobacteria (Chapter III), and to see how well the toxin-producing cyanobionts follow the general patterns of symbiont selection.

The specific aim in Chapter I was to detect whether cyanobacterial toxin microcystin is produced *in situ* in lichen symbiosis, and to isolate and identify the producer. In Chapter II the aim was to untangle the geographic distribution of the phenomenon in a global scale in several different cyanolichen genera. In Chapter III the focus was on one genus, *Nephroma*, to more thoroughly describe the phylogeny and cyanobiont selection patterns within one cyanolichen genus that included species that house microcystin-producing *Nostoc* strains. The aim in Chapter IV was to further elucidate relationships between toxin production and fungal phylogeny, and to find possible correlations between cyanobacterial toxin production, fungal secondary chemistry, and patterns of symbiont selection.

3. Material and methods

Lichen specimens

The focus of taxon sampling was on Peltigeralean lichens, a great majority of which are known to contain cyanobacterial symbionts of the genus *Nostoc*. Also other relatively abundant, common and widespread macrolichens were analyzed, including many species of *Leptogium, Lobaria*, *Nephroma*, *Peltigera*, *Pseudocyphellaria*, and *Sticta*. Lichen specimens were collected from numerous locations in different parts of the world and from many different habitat types by myself, co-authors Jouko Rikkinen and Katja Fedrowitz, and by kind colleagues (Table 1).

Toxin analyses

The microcystins and nodularins were detected by liquid chromatography-mass spectrometry (LC-MS). The analyses were performed with an Agilent 1100 Series LC/MSD Trap System high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA.), which has an XCT Plus model ion trap as a mass detector. Toxins were identified by LC-MS/MS according to their microcystin characteristic protonated molecular ions $[M+H]^+$, fragment ion spectra of the $[M+H]^+$, and in Chapter I also by by-product ion fragmentation (MS^3). The total microcystin and nodularin concentrations were approximated with MC-RR (Alexis), MC-LR, and Nod-R standards (gifts to K. Sivonen from Z. Grzonka, University of Gdansk, Gdansk, Poland).

Genetic markers

Fungal internal transcribed spacer

The internal transcribed spacer (ITS, ITS1-5.8S-ITS2) has been widely used in phylogenetic studies of Peltigeralean fungi (Ekman & Jørgensen 2002; Lohtander *et al.* 2002; Goffinet *et al.* 2003; Miadlikowska *et al.* 2003; Piercey-Normore *et al.* 2006; Otalora *et al.* 2008; O'Brien *et al.* 2009; Serusiaux *et al.* 2009, 2011; Wei *et al.* 2009). This region has proven to be handy for identifying closely related species in Peltigeralean fungi (papers III and IV), and it was recently formally proposed as the primary fungal barcode marker for *Fungi* (Schoh *et al.* 2012). All the primers used in this study are listed in Table 2.

Cyanobacterial 16S rRNA gene and tRNA^{Leu} (UAA) intron

The 16S rRNA gene is the primary marker in cyanobacterial phylogenetics, and it has been widely used to clarify taxonomic affinities of Nostocalean cyanobacteria (e.g. Woese *et al.* 1985; Lyra *et al.* 2001; Rikkinen *et al.* 2002; Oksanen *et al.* 2004a; Svenning *et al.* 2005; Papaefthimiou *et al.* 2008; Han *et al.* 2009; Olsson *et al.* 2012). However, the region is quite conserved and therefore not convenient when working with closely related lichen-symbiotic *Nostoc* genotypes.

Cyanobacterial tRNA^{Leu} (UAA) intron (trnL) is a self-splicing group-I intron present in most cyanobacteria (Paquin *et al.* 1997; Fewer 2001). The intron consists of conserved regions and more variable loops and hairpin structures (Costa *et al.* 2002). It often also includes substantial length variation due to the different numbers of repeat motifs in the 'hypervariable' p6b region (Costa *et al.* 2002). trnL is relatively short, very easily amplified, variable enough and therefore commonly used in studies concerning the lichen-symbiotic *Nostoc* (Paulsrud *et al.* 1998, 2000; Rikkinen 2004; Summerfield & Eaton-Rye 2006; Fedrowitz *et al.* 2011). However, because of its possibly polyphyletic origin and ambiguous

Table 1. The collection localities, the number of different cyanolichen genera collected from each location, and collector(s) of lichen specimens analysed. The locations are listed according to the total number of analysed lichen specimens.

Collection place	Cyanolichen genera	Collector(s)
Southern Finland	9	K. Fedrowitz, U. Kaasalainen, J. Rikkinen
Taita Hills, Kenya	8	J. Rikkinen
Hunan, China	4	J. Rikkinen
Bariloche, Argentina	7	K. Fedrowitz
Lapland (Northern Finland and Sweden)	5	K. Fedrowitz, J. Rikkinen
Scotland	8	K. Fedrowitz
Oregon, USA	7	U. Kaasalainen, J. Rikkinen
Hokkaido, Japan	3	A. Frisch & G. Thor
California, USA	10	U. Kaasalainen
Norway	8	U. Kaasalainen, P. Larsson
Svalbard	3	J. Rikkinen
Gran Canaria, Canary Islands	2	J. Rikkinen
Quebec, Canada	1	H. Coffey & C. Freebury
Yunnan, China	1	T. Ahti
Hawaii, USA	1	Randolph & Weber
Tibet, China	1	Obermayer

p6b-region the entire trnL cannot be used in phylogenetic reconstruction (Rudi & Jakobsen 1997, 1999; Rudi *et al.* 2002; Oksanen *et al.* 2004b). However, it is very applicable for assessing symbiont selectivity patterns inside certain closely related groups (Olsson *et al.* 2012).

Microcystin synthetase gene E

In order to determine the presence or absence of the microcystin gene cluster in a cyanobacterial genome, we amplified the microcystin synthetase gene E (*mcyE*) or the corresponding nodularin synthetase gene F (*ndaF*). These genes are involved in the synthesis of Adda and the formation of the bond between Adda and D-glutamate (Tillett *et al.* 2000; Rouhiainen *et al.* 2004), which are essential for the toxicity of the microcystin and show very little variation between microcystin variants (Sivonen & Jones 1999). These genes are also believed to be unaffected by horizontal gene transfer (Tillett *et al.* 2000; Rantala *et al.* 2004; Jungblutt & Neilan 2006). Consequently the *mcyE* gene is expected to be less variable than the genes coding other more variable amino acids in the microcystin molecule.

Data analysis

Sequences were edited and aligned manually using BioEdit v7.0.9.0 (Hall 1999) and Phyde v0.995 and 0.996 (Müller *et al.* 2005).

Table 2. Primers used in this study. A, the primer was used in amplification; S, the primer was used in sequencing.

Region	Primer name	Sequence (5'-3')		Chapters	Reference
ITS	ITS 1F	tccgtaggtgaacctgcgg	A&S	III, IV	White *et al.* 1990
ITS	ITS 4R	tcctccgcttattgatatgc	A&S	III, IV	White *et al.* 1990
ITS	ITS 5F	ggaagtaaaagtcgtaacaagg	S	III, IV	White *et al.* 1990
16S	106F	cggacgggtgagtaacgcgtga	S	II	Nübel *et al.* 1997
16S	23S30R	cctcgcctctgtgtgcctaggt	A	I, II	Lepere *et al.* 2000
16S	27F	agagtttgatcmtggctcag	A&S	I, II	Wilmotte *et al.* 1993
16S	359F	ggggaatyttccgcaatggg	A	I	Nübel *et al.* 1997
16S	781Ra/b	gactacaggggtatctaatcccwtt	A	I	Nübel *et al.* 1997
16S	pCR	cccactgctgcctcccgtag	S	II	Edwards *et al.* 1989
16S	pDF	cagcagccgcggtaatac	S	I, II	Edwards *et al.* 1989
16S	pDR	gtattaccgcggctgctg	S	I	Edwards *et al.* 1989
16S	pEF	aaactcaaaggaattgacgg	S	I, II	Edwards *et al.* 1989
trnL	trnL inF	agaattcggtagacgcwrcggactt	S	III	Paulsrud & Lindblad 1998
trnL	trnL outF	ggaattcggggrtrtggygraat	A	III	Paulsrud & Lindblad 1998
trnL	trnL outR	tcccggggryrgrgggactt	A	III	Paulsrud & Lindblad 1998
trnL	trnL UFII	ggtagacgctacggactt	S	III	III*
trnL	trnL UR	gggacttgaacccacacgacc	S	III	Fedrowitz *et al.* 2011*
mcyE	mcyE F2	gaaatttgtgtagaaggtgc	A&S	I, II	Rantala *et al.* 2004
mcyE	mcyE R4	aattctaaagcccaaagacg	A&S	I, II	Rantala *et al.* 2004
mcyE	mcyEF dgn	tcaacaggaaayccyaaaggag	A	II	Fewer *et al.* 2007
mcyE	mcyER dgn	gaccaaccatcdraaatgatatggtgcat	A	II	Fewer *et al.* 2007

* modified from Paulsrud & Lindblad (1998)

The Bayesian analyses were performed with MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001), maximum likelihood analyses with PAUP* v4 (Chapter I; Swofford 1998) and Garli v2.0 (Chapter III; Zwickl 2006), the final maximum parsimony and neighbor-joining analyses with PAUP* v4, and some initial neighbor-joining analyses with MEGA5 (Tamura *et al.* 2011). The best-fitting nucleotide substitution models for Bayesian analyses were chosen with jModelTest v0.1.1 (Posada 2008), and the stationarity of the MCMC search was determined with Tracer v1.4 (Rambaut & Drummond 2007). Consensus topologies were compiled and drawn using TreeGraph2 (Stöver & Müller 2010) and occasionally with FigTree v.1.3.1 (Rambaut 2006). The haplotype network was constructed with Network 4.6.0.0 (Bandelt *et al.* 1999) using median-joining method, and the ordinations performed with PC-ORD 5.33 (McCune & Mefford 2006).

Methodological approaches

Chapter I

Cyanobacterial toxins were detected from the tripartite lichen *Peltigera leucophlebia* by analyzing a pooled sample of cephalodia by LC-MS. The symbiotic *Nostoc* strain was isolated and cultured, and the produced microcystins were analyzed both qualitatively and quantitatively. The cyanobacterial 16S rRNA and *mcyE* genes were amplified, cloned, and sequenced from the same pooled sample of cephalodia, and amplified and sequenced from the cultured strain (UK18). Phylogenetic trees were inferred from the 16S rRNA gene sequences by using neighbor-joining, maximum parsimony, and maximum likelihood methods.

Chapter II

DNA was extracted from 803 cyanolichen specimens, mainly representing different genera in the order Peltigerales, collected from five different continents. The extractions were screened for the cyanobacterial *mcyE* gene by PCR. The detected *mcyE* genes were sequenced and the lichen specimens with the gene analyzed by LC-MS to detect the cyanobacterial toxins microcystin and nodularin. Toxin structures were identified and, in selected specimens, also the concentrations measured. The cyanobacterial 16S rRNA gene was amplified and sequenced, when possible, from the same DNA extraction as the *mcyE* gene. The *mcyE* and 16S rRNA genes were used to infer Bayesian phylogenies.

Chapter III

The diversity of the genus *Nephroma* mycobionts were studied by inferring their phylogeny from fungal ITS sequences using Bayesian and maximum likelihood methods. From selected specimens the variation in fungal secondary chemistry was analyzed with TLC. In addition cyanobacterial trnL intron sequences were used to construct a haplotype network to describe the genetic diversity of the associated cyanobionts.

Chapter IV

The distribution of toxin-producing cyanobacteria in many lichen species included in paper II was studied further by overlaying the distribution of *mcyE* gene and/or toxin-containing specimens on fungal ITS phylogenies inferred for the sections *Horizontales*, *Peltigera*, and *Polydactylon* of *Peltigera* and the genus *Nephroma*, respectively. From selected specimens the variation in fungal secondary chemistry was analyzed with TLC. Correlations between the presence of different microcystin variants, species identities of lichen specimens, and geographic origin were analyzed and illustrated with NMS-ordination.

4. Main results and their interpretation

The main finding of this thesis was that hepatotoxic microcystins are produced *in situ* in lichen symbiosis by symbiotic cyanobacteria (I). Microcystins and nodularin are produced commonly in many different lichen genera all over the world and this production is linked to remarkable chemical and genetic diversity (II). Symbiont selection in the lichen genus *Nephroma* is more specific locally than globally and differs between bi- and tripartite species of the genus (III). The distribution of toxin-producing cyanobacteria in lichens is not uniform but concentrates into certain taxonomic groups. Toxin-producing cyanobionts are especially common in some species of the Lobariaceae, Nephromataceae, and Peltigeraceae (Peltigerales, Ascomycota), and the diversity of microcystin structures correlates with the genetic identities of *Nostoc* symbionts in these lichens (IV).

Cyanobacterial toxins in lichens and potential implications to grazers

Microcystin production was first shown in the cephalodia of the tripartite lichen *Peltigera leucophlebia*, and its isolated and cultured *Nostoc* cyanobiont (I). The production of hepatotoxic microcystins and nodularin was later detected in numerous other lichen species belonging to many different genera and collected from five different continents (II). This shows that the production of microcystins by symbiotic cyanobacteria in lichens is a common phenomenon worldwide, and indicates that the cyanotoxin nodularin, previously only known from the aquatic genus *Nodularia*, may also be produced by lichen-symbiotic *Nostoc*. The production of nodularin has very recently also been detected in a symbiotic *Nostoc* strain isolated from the root of the cycad *Macrozamia* (Gehringer *et al.* 2012).

During this study cyanobacterial toxins were searched from lichen specimens representing 21 different lichen genera. Ten of these had representatives containing the *mcyE* gene and four were also found to contain the cyanobacterial toxins microcystin or nodularin, which accounted 12 and 5 percent off all analyzed lichen specimens, respectively (II, IV). In Peltigeralean lichens hepatotoxin-producing cyanobacterial symbionts were most common in specimens of Lobariaceae, Nephromataceae, and Peltigeraceae (IV). In *Peltigera* toxic cyanobacteria were particularly common in *P. degenii* and *P. membranacea* (section *Peltigera*), and different taxa in the species complex formed by *P. occidentalis*, *P. dolichorhiza*, *P. hymenina,* and *P.* sp. E (section *Polydactylon*). In *Nephroma* toxic cyanobacteria were most common in *N. parile* and *N. cellulosum*. Species of *Leptogium* and *Pseudocyphellaria*, and those of *Peltigera* section *Phlebia* seem to usually host *Nostoc* genotypes that do not commonly produce microcystin or nodularin (IV).

The measured toxin concentrations in lichen thalli varied from trace amounts to over 0.2 mg g^{-1} dry weight (dw) of microcystin, and up to 0.06 mg g^{-1} dw of nodularin (II). Microcystins and nodularins, usually assimilated from contaminated drinking water, have caused deaths of wild and domestic animals (Sivonen 2009), and there is also strong evidence that these toxins can reduce the fitness and reproduction of aquatic invertebrates (e.g. Rohrlack *et al.* 2001; Kozlowsky-Suzuki *et al.* 2003). The LD_{50} value for mice varies between 50–300 µg kg^{-1} body weight (Sivonen & Jones

1999) so theoretically approximately one gram of the most toxic lichen would contain enough microcystins to kill half of an experimental 1 kg of mice.

In the isolated and cultured *Nostoc* strains the concentration of microcystin varied from 0.2 mg g^{-1} dw up to almost 5 mg g^{-1} dw (I, II). Microcystin concentrations up to over 7 mg g^{-1} dw and nodularin concentrations up to 18 mg g^{-1} dw of have been reported from aquatic cyanobacterial strains (Sivonen & Jones 1999). The microcystin concentrations previously detected from *Nostoc* strains CENA88, IO-102-I, and 152 have varied from 0.05 to 2 mg g^{-1} dw (Oksanen *et al.* 2004a; Genuário *et al.* 2010). This shows that the concentrations now measured from lichen-symbiotic strains are well within the average range for toxin-producing cyanobacteria in general, but somewhat higher than those previously reported from *Nostoc* strains.

The distribution of cyanobacterial toxins in lichens revealed that there are clear differences in the frequency of these compounds between different geographical regions and lichen species (II, IV). As a whole, the production of hepatotoxins appeared to be most frequent in regions with temperate, humid climates, such as Scotland, Norway, and Oregon (II), where also the grazing pressure by mollusks and other invertebrates can be expected to be relatively high. On the other hand, microcystins were found to be relatively rare in cyanolichens collected from tropical montane forests of Kenya, and from warm temperate to subtropical forests of Hunan Province in China (II), where lichen herbivory could be expected to be at least as severe. This is in line with the results by Hrouzek *et al.* (2011) who reported that the cytotoxicity of terrestrial *Nostoc* strains varies between different climates and geographic regions. In addition, in some cases geographical variation was detected in the toxicity of individual lichen species (Fig. 3). For example, in *Peltigera degenii* and *P. membranacea* some proportion of lichens from all locations contained cyanobacterial toxins, while in *Nephroma parile* toxins were present in all specimens collected from Norway and Scotland, but not in lichens collected from other regions.

Scotland was by far the most interesting region with respect to microcystin frequency. Almost 60 % of all cyanolichen specimens analysed from Scotland contained the *mcyE* gene, and microcystins were detected in no less than 18 % of all specimens (II). The frequency of specimens with the *mcyE* gene present but no toxins detected was curiously high, and also specimens with ambiguous *mcyE* sequences were relatively frequent (II). Also the spectrum of microcystin variants was distinctive: while in most other locations *Peltigera* and *Nephroma* specimens typically had contrasting toxin spectra, in Scotland all the lichens studied were found to contain quite similar toxin structures (II, IV).

Many animals, including for example molluscs, insects, and oribatid mites, but also voles and snub-nosed monkeys feed on lichens (Li 2007; Gauslaa 2008; Fischer *et al.* 2010; Nybakken *et al.* 2010; Fröberg *et al.* 2011), and damage due to anthropod feeding is quite common on *Peltigera* thalli, for example. Mollusc grazing has even been suggested to be a limiting ecological factor for some cyanolichen species in the boreal rainforests of western Norway (Asplund & Gauslaa 2008). Certain lichen species are important winter feed for reindeer and caribou, and for example in northern Finland *Cladonia* species and some other green algal lichens are heavily grazed (Danell *et al.* 1994; den Herder *et al.* 2003). The nitrogen content of lichens with green algae photobionts is so low, that reindeer fed solely with *Cladonia* tend to lose weight (Storeheier *et al.* 2002). The nitrogen content of cyanolichens can be substantially higher (Rai 2002), which would indicate better nutritional value compared to green algal lichens. However, reindeer distinctly avoid eating cyanolichens even during

starvation, and the only previously proposed explanation for this phenomenon is the apparently poor digestibility of some cyanolichen species (Hofmann 1989; Storeheier *et al.* 2002).

The results of this study provide strong circumstantial evidence that reindeer and other lichen herbivores could easily be exposed to hepatotoxic microcystins, if they were to indiscriminately feed on terricolous cyanolichens. Thus, the presence of cyanobacterial metabolites may be one reason why some herbivores avoid eating cyanolichens. It may well be that even the possible presence of cyanobacterial hepatotoxins, or other variably effecting chemicals produced by cyanobacteria (Sivonen 2009), is enough to make cyanobacterial lichens unappealing to herbivores. In this study no obvious connection was found between the presence of cyanobacterial toxins and the secondary chemistry of the lichen species; while some lichens without lichen compounds commonly contained cyanobacterial toxins, other species did not (IV). It is difficult to perceive how lichen grazers could accurately detect the

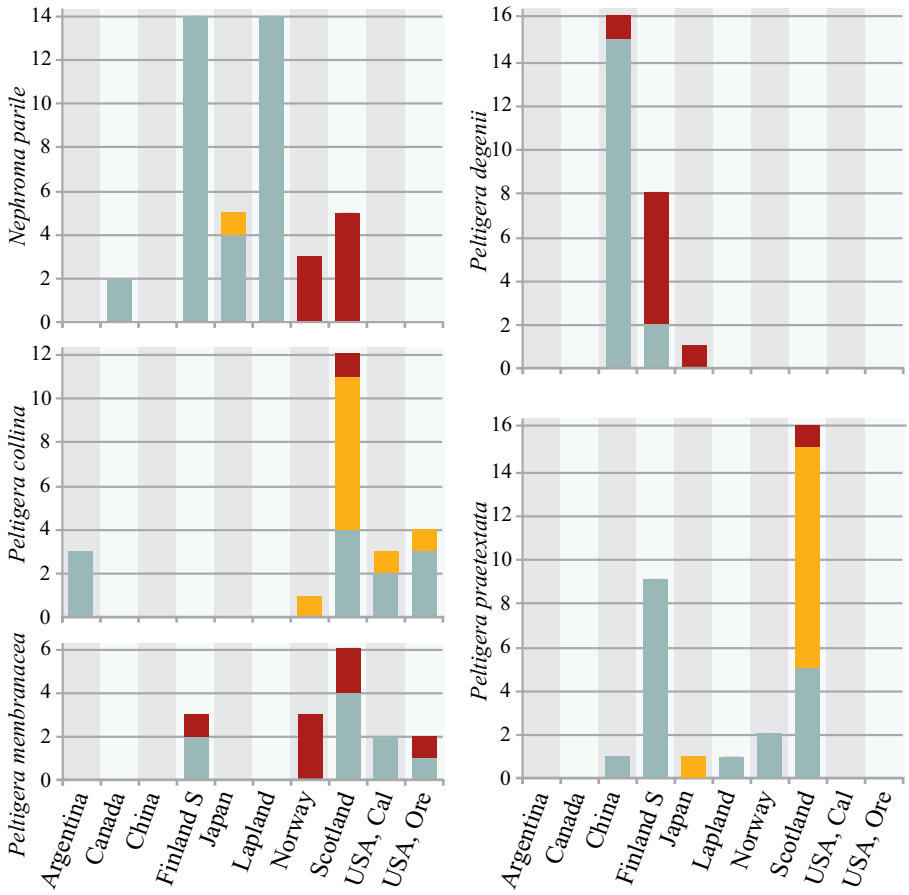

Figure 3. The frequency of the *mcyE* gene and cyanobacterial toxins in five cyanolichen species in different geographical regions. The vertical axis shows the number of lichen specimens analyzed. The red proportion of the bar refers to the specimens with both the *mcyE* gene and detected toxins, yellow to specimens with the *mcyE* gene but no detected toxins, and grey to specimens with neither the *mcyE* gene nor detected toxins.

presence or absence of microcystins in individual lichen thalli and concurrently many of them may avoid eating cyanobacterial lichens altogether. The 'warning sign' for the presence of potentially dangerous cyanobacterial metabolites could be some volatile compound, like geosmin (e.g. Izaguirre *et al.* 1982; Giglio *et al.* 2008), which is commonly produced by cyanobacteria and presumably easily detected by the acute senses of herbivores such as reindeer. In any case, it can be safely concluded that the toxins produced by lichen-symbiotic cyanobacteria pose a real threat to lichen feeders. They may also act as grazing deterrents provide some protection to the thallus. As a whole, the actual consequences of exposure to microcystins for different grazers and the faith of these toxins in the food chain deserve to be studied in much more detail.

Cyanobacterial diversity and the lichen-symbiotic way of life

The lichen thalli analysed in this study were found to contain a true plethora of different microcystins. Over 50 different chemical variants were detected, and many of these have only rarely been reported from aquatic cyanobacteria. In microcystins *D*-Ala in position one is usually highly conserved, but in lichen-symbiotic cyanobacteria, it is often replaced by *D*-Leu. Furthermore, the amino acid Adda, unique to microcystin and nodularin, is often in the acetylated form (ADMAdda; Fig. 2). In fact, all but two lichen specimens only had microcystins with one or both of these interesting modifications (I, II). As already mentioned the *mcyE* gene can be expected to vary less than genes coding other more variable amino acids in the molecule, and it does not necessarily reflect the diversity of microcystin variants the cyanobacterium is producing. The variability of the *mcyE* gene is thus partially independent from the variation of the chemical structures and quite intriguing as such.

All lichens have the potential of reproducing and dispersing symbiotically by thallus fragmentation, and in addition several lichens produce specific symbiotic propagules for the combined dispersal of both or all symbionts (Büdel & Scheidegger 2008). When packaged into symbiotic propagules, the *Nostoc* symbiont population is commonly reduced to only a few trichomes and they invariably experience a genetic bottleneck (Bright & Bulgheresi 2010; Mandel 2010; Fedrowitz *et al.* 2011). The very close association with a symbiotic fungus can be expected to promote the evolution of different traits than in non-symbiotic cyanobacteria: for example the genome of the cyanobacterial symbiont of the water fern *Azolla* has eroded drastically during prolonged symbiosis (Ran *et al.* 2010). Also the exceptionally high diversity of *mcyE* genes and peculiar toxin structures now observed in lichen-symbiotic cyanobacteria may be partly explained by the effects of re-occurring extreme population bottlenecks and other population-shaping effects that characterize lichen symbioses (Rikkinen *et al.* 2002; Rikkinen 2003; Fedrowitz 2011). Interestingly, a very high proportion of all analyzed specimens of *Peltigera collina* and *P. praetextata*, both of which are symbiotically dispersing lichen species, had the *mcyE* gene but no toxins detected. As a whole, however, the *mcyE* sequence data did not provide evidence of random genetic drift but rather supported the hypotheses of purifying selection (Hartl & Clark 1997; Fay & Wu 2003). This would indicate that even though the *mcyE* gene of lichen-symbiotic *Nostoc* has gone through substantial diversification, natural selection seems to have favoured and maintained certain genotypes.

General patterns of symbiont selection and microcystin-producing cyanobacteria

In the cyanobacterial 16S rRNA phylogeny (II) the lichen-symbiotic *Nostoc* genotypes were grouped into two previously described

lineages (Rikkinen *et al.* 2002; O'Brien *et al.* 2005; Myllys *et al.* 2006). *Nostoc* genotypes A–F, identified mainly from lichen species of the *Nephroma* guild, were clearly distinct from another well supported group formed by genotypes G–L, identified mainly from lichen of the *Peltigera* guild. Also the toxins produced by the cyanobacteria grouped in a similar manner: all the *Nostoc* cyanobionts of *Nephroma* species had microcystin variants with the amino acid ADMAdda in the fifth position, while the majority of *Peltigera* specimens contained toxins of a different type, with the amino acid leucine in the first position of the microcystin structure (IV).

In genus *Nephroma* (III), several species associated with different *Nostoc* genotypes in different parts of their range, leading to relatively higher selectivity locally as compared to lower selectivity globally. It was concluded that reproduction by fungal spores and the occasional breakdown of symbiotic propagules and subsequent re-association of one or both symbionts have given rise to a geographical mosaic of symbiont associations (Thompson 2005). Several features in the association mosaic of *Nephroma* suggest that once a certain combination of symbionts has been successfully established in a certain area, this particular combination has a tendency to become regionally dominant (III). Also the cyanobiont selection of bi- and tripartite species of the genus *Nephroma* is markedly different (Lohtander *et al.* 2003). This was seen both with trnL (III) and in the 16S rRNA gene tree (II) where the cyanobionts of *Nephroma arcticum* grouped together with those of *Peltigera* species and not with other *Nephroma* cyanobionts. When the trnL and ITS data obtained from the genus *Nephroma* are compared to the toxin and *mcyE* gene data, the three *mcyE*-containing specimens of *Neproma cellulosum* can be seen to have identical trnL genotypes, which differ from the trnL sequence from the fourth specimen that lacked the *mcyE* gene. In case of *Nephroma parile* microcystins were detected from all specimens collected from Norway and Scotland which all also had the same trnL genotype. Interestingly, however, several *Nephroma parile* specimens representing the same fungal ITS and cyanobacterial trnL genotype from Finland and Sweden did not contain the *mcyE* gene nor toxins (Fig. 3).

On a wider taxonomic scale, the phylogenetic tree constructed of the 16S rRNA gene sequences of the *mcyE* gene containing lichen cyanobionts (II) shows that mosaic-like symbiont selection patterns similar to those described for the genus *Nephroma*, are also evident in *Peltigera*. For example *Peltigera membranacea* associates with at least three different *Nostoc* 16S genotypes: one in Scotland (shared with *P. hymenina*), the second in Finland (shared with *P. degenii*, *P. occidentalis*, and one species of section *Polydactylon* from Finland, Swedish Lapland, Oregon USA, and Japan), and the third in Norway and Oregon (shared with a further species in the section *Polydactylon*, also from Oregon) (II, IV). The two symbiotically dispersing species *Peltigera collina* and *P. praetextata* each associate mainly with one *Nostoc* genotype, and in some cases with additional rare genotypes none of which are shared with any other lichen species (II). This indicates that also in *Peltigera* the vertical transmission of *Nostoc* symbionts may promote high selectivity (e.g. Beck *et al.* 2002; Otálora *et al.* 2010; Fedrowitz *et al.* 2011), although such a pattern was not clear over wide geographic scales in *Nephroma* (III).

The *Nostoc* 16S rRNA genotypes in lichens of the *Peltigera* guild formed several well supported groups. One group included the cyanobionts of *Nephroma arcticum* and *Leptogium lichenoides*, the second mainly cyanobionts of *Peltigera collina*, the third mainly cyanobionts of *P. praetextata*, and the fourth cyanobionts of *P. degenii*, *P. membranacea*, and various species of *P.* section *Polydactylon* (II). This is in congruence with previous results in which the *Nostoc* cyanobionts of *Peltigera*

degenii, *P. membranacea*, and *P. neopolydactyla* have been found to belong to a different phylogenetic group than the cyanobionts of *P. praetextata* (Myllys *et al*. 2007). Even though the phylogenies inferred from 16S rRNA and *mcyE* genes were not fully congruent (II), a group including most specimens of *Peltigera degenii*, *P. membranacea*, and *P. occidentalis* was also present in the *mcyE* gene tree (II). These results suggest that *Peltigera degenii*, *P. membranacea* and *P. occidentalis* can often house identical cyanobionts and they are very likely to belong to the same functional guild. This guild appears to be widely distributed on the Northern Hemisphere, and it does not normally include species such as *P. praetextata* and *P. collina*.

Cyanobacterial toxins were found to be present in some species of several lichen genera, and the toxin structures were found to correlate with the genus of the fungal host. There were also clear differences between geographical locations and some of this variability seemed to be indirectly related to the climatic factors.

5. Final remarks and future considerations

The results of this study show that cyanobacterial hepatotoxins, previously primarily associated with aquatic cyanobacterial blooms, are also commonly present in lichen symbioses in terrestrial environments. The microcystin concentrations detected from many cyanolichen species in several different genera and from different parts of the world are high enough to pose a potential risk to lichen grazers. On the other hand they may also provide some cyanolichens with an additional defence against herbivory. The actual consequences of cyanobacterial toxins to lichen grazers and the faith of these toxins in the food chain wait to be examined in further studies. Because it seems quite unlikely that the herbivores could actually detect the microcystins or related compounds, it would be intriguing to identify the actual signal compounds which help lichen grazers to avoid cyanolichens.

The symbiont selection of lichen genus *Nephroma* was found to be more specific locally than globally and to differ between bi- and tripartite members of the genus. We also got some preliminary evidence that symbiont selection in the genus *Peltigera* exhibits similar mosaic-like patterns as in *Nephroma*, even though the majority of cyanobionts in these two genera belong to different groups of *Nostoc*. In order to achieve a better understanding of the principles of symbiont selection and population dynamics in cyanolichens it would be essential to continue thorough and extensive analyses of cyanobiont selection in other lichen genera and in additional habitat types and geographical regions. This would undoubtedly reveal many currently unrecognized functional guilds and increase our knowledge of lichen systematics, ecology, and biogeography.

The rather sporadic and uneven distribution of the toxic-producing *Nostoc* genotypes among the studied lichens still indicates that toxin-production *per se* is usually not the primary reason why certain *Nostoc* genotypes are selected by certain fungal hosts. Their distribution seems to more reflect general patterns of symbiont selection rather than specific

favoring of toxin-producing cyanobacteria. In any case, the many specific associations observed between particular mycobiont and cyanobiont genotypes were far from random, and many of them may actually be more complex and finely regulated than we can even presently imagine.

The resuts of this study exemplify the vast genetic and chemical diversity harboured in lichen symbiosis, and many intriguing differences between symbiotic and free-living cyanobacteria. Many such differences are undoubtedly closely related to the symbiotic life-cycle, often very different from that experienced by free-living cyanobacteria, and the fact that fungal hosts are bound to prefer cyanobcterial abilities and traits that are not necessarily of primary importance to non-symbiotic cyanobacteria. The now unravelled diversity is also promising from the biotechnological point of view. Researchers are constantly looking for new bioactive compounds for different purposes, and promising candidates with e.g. anticancer and antibiotic activities have been detected from both free-living cyanobacteria and lichens (Burja *et al.* 2001; Oksanen 2006). While lichenologists have traditionally been quite focused on the substances produced by lichen-forming fungi, these results once again emphasize that the bioactive prospects of cyanobacterial and other photosynthetic symbionts should not be overlooked.

I believe that the patterns of symbiont selection, here exemplified in the distributions of trnL genotypes in *Nephroma* and those of toxin-producing cyanobionts in *Peltigera* reflect fundamental processes of lichen symbiosis, and very similar patterns will surely be detected also in other cyanolichen groups.

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