Cyanobacteria in symbiosis with boreal forest feathermosses

from genome evolution and gene regulation to impact on the ecosystem

Denis Warshan

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Abstract

Among dinitrogen (N_2)-fixing some cyanobacteria can establish symbiosis with a broad range of host plants from all plant lineages including bryophytes, ferns, gymnosperms, and angiosperms. In the boreal forests, the symbiosis between epiphytic cyanobacteria and feathermosses *Hylocomium splendens* and *Pleurozium schreberi* is ecologically important. The main input of biological N to the boreal forests is through these cyanobacteria, and thus, they greatly contribute to the productivity of this ecosystem. Despite the ecological relevance of the feathermoss symbiosis, our knowledge about the establishment and maintenance of cyanobacterial-plant partnerships in general is limited, and particularly our understanding of the feathermoss symbiosis is rudimentary.

The first aim of this thesis was to gain insight on the genomic rearrangements that enabled cyanobacteria to form a symbiosis with feathermosses, and their genomic diversity and similarities with other plant-symbiotic cyanobacteria partnerships. Genomic comparison of the feathermoss isolates with the genomes of free-living cyanobacteria highlighted that functions such as chemotaxis and motility, the transport and metabolism of organic sulfur, and the uptake of phosphate and amino acids were enriched in the genome of plant-symbiotic cyanobacteria.

The second aim of this PhD study was to identify cyanobacterial molecular pathways involved in forming the feathermoss symbiosis and the regulatory rewiring needed to maintain it. Global transcriptional and post-transcriptional regulation in cyanobacteria during the early phase of establishment of the feathermoss symbiosis, and after colonization of the moss were investigated. The results revealed that the putative symbiotic gene repertoire includes pathways never before associated with cyanobacteria-plant symbioses, such as nitric-oxide sensing and regulation, and the transport and metabolism of aliphatic sulfonate.

The third aim was to explore the role of the cyanobacterial community in contributing to the temporal variability of N_2-fixation activity. Results from a field-study showed that temporal variation in N_2-fixation rates could be explained to a high degree by changes in cyanobacterial community composition and activity. In particular, the cyanobacteria belonging to the genus *Stigonema* - although not dominating the community- appeared to be the main contributors to the N_2-fixation activities. Based on this result, it is suggested that this genus is responsible for the main input of N in the boreal forest ecosystems.

The last aim was to understand how the relationship between cyanobacterial community composition and N_2-fixation activity will be affected by climatic changes such as, increased temperature (11°C compared to 19°C) and CO_2 level (500 ppm compared to 1000 ppm). Laboratory experiments highlighted that 30 weeks of combined elevation of temperature and CO_2 resulted in increased N_2-fixation activity and moss growth rates. The observed increases were suggested to be allocated to reduced cyanobacterial diversity and changes in community composition, resulting in the dominance of cyanobacteria adapted to the future abiotic condition.

Keywords: *Cyanobacteria, Feathermosses, Symbiosis, Boreal forest, Gene flow, Proteogenomic, Transcriptomic, Community structure and composition, Dinitrogen fixation.*

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My father once told me:
“Your mother, your brother, you and me are like arms and legs of the same body. Moving forward together”. I was always wondering who is the “head”. Apparently one can get a PhD without one….
To my family.

Sammanfattning

Kvävefixerande cyanobakterier lever i symbios med ett brett spektrum av värdväxter såsom mossor, ormbunkar, gymnospermer och angiospermer. I nordliga barrskogar är symbios mellan epifytiska cyanobakterier och två arter av fjädermossa, husmossa (*Hylocomium splendens*) och väggmossa (*Pleurozium schreberi)* ekologiskt viktiga. Denna symbios är barrskogens huvudsakliga källa av biologiskt fixerat kväve och bidrar i hög grad till ekosystemets produktivitet. Trots den ekologiska betydelsen av fjädermossornas symbios är vår kunskap om etableringen och underhållet av partnerskap mellan cyanobakterier och växter i allmänhet begränsad, och i synnerhet är vår förståelse av fjädermossornas symbios rudimentär.

Det första syftet med denna avhandling var att öka kunskapen om de genomiska förändringar som möjliggjort för cyanobakterier att bilda symbios med fjädermossor samt om genomiska skillnader och likheter med andra partnerskap mellan cyanobakterier och växter. Jämförelser av genomet hos ett antal isolat av cyanobakterier i symbios med fjädermossor med det hos frilevande cyanobakterier belyste att funktioner såsom kemotaxis och rörlighet, transporten och omsättningen av organiskt svavel samt upptagningen av fosfat och aminosyror var anrikade i genomet hos växt-symbiotiska cyanobakterier.

Avhandlingens andra delprojekt syftade till att i cyanobakterierna identifiera de molekylära vägar som är involverade i bildningen av symbios med fjädermossor och de regulatoriska förändringar som krävs för att upprätthålla den. Detta undersöktes genom vittomfattande analys av transkriptionell och posttranskriptionell reglering i cyanobakterier under den tidiga fasen av etablering av symbios med fjädermossa och efter koloniseringen av mossan. Resultaten visade att vissa funktioner som aldrig tidigare förknippats med symbios mellan cyanobakterier och växter troligen ingår i repertoaren för symbios-relaterade gener, exempelvis avkänning och reglering av kväveoxid samt transporten och omsättningen av alifatiskt sulfonat.

Det tredje syftet var att undersöka cyanobakteriesamhällets roll för att bidra till variationen i kvävefixeringen över tid. Resultat från en fältstudie visade att variationer i kvävefixeringsaktiviteter över tid i hög grad kunde förklaras av förändringar i sammansättningen av och aktiviteten i cyanobakteriesamhället. Det visade sig att i synnerhet cyanobakterier tillhörande släktet *Stigonema* gav det största bidraget till de kvävefixerande aktiviteterna, om än de

inte dominerade i cyanobakteriesamhället. På grundval av detta resultat föreslås att detta släkte ansvarar för det huvudsakliga bidraget av kväve i de nordliga barrskogarnas ekosystem.

Det sista syftet var att förstå hur förhållandet mellan cyanobakteriesamhällets sammansättning och kvävefixeringsaktivitet kommer att påverkas av klimatförändringar såsom förhöjda temperaturer och ökade koldioxidhalter. Laboratorieförsök visade att 30 veckor vid högre temperatur (19^{o}C jämfört med 11^{o}C) och koldioxidhalt (1000 ppm jämfört med 500 ppm) orsakade ökad kvävefixeringsaktivitet och tillväxthastighet hos mossan. De observerade ökningarna föreslås att orsakas av minskad mångfald av cyanobakterier samt förändringar i sammansättningen av cyanobakteriesamhället, vilka resulterar i dominans av cyanobakterier som är anpassade till framtida abiotiska förhållanden.

List of publications

This thesis is based on the following papers:

I. **Warshan D**, Liaimer A, Pederson E, Sea-Yong K, Shapiro N, Woyke T, Altermark B, Pawlowski K, Weyman P, Dupont CL, Rasmussen U. Shared and divergent genomic changes associated with the evolutionary transitions of *Nostoc* to a plant symbiont. *Manuscript*.

II. **Warshan D**, Espinoza JL, Stuart RK, Richter RA, Sea-Yong K, Shapiro N, Woyke T, Kyrpides N, Barry K, Singan V, Lindquist E, Ansong C, Purvine SO, Brewer H, Weyman P, Dupont CL, Rasmussen U. (2017). Feathermoss and epiphytic *Nostoc* cooperate differently - expanding the spectrum of plant-cyanobacteria symbiosis. ISME J, doi:10.1038/ismej.2017.134

III. **Warshan D**, Bay G, Nahar N, Wardle DA, Nilsson M-C, Rasmussen U. (2016). Seasonal variation in *nifH* abundance and expression of cyanobacterial communities associated with boreal feather mosses. ISME J, 10: 2198–2208.

IV. **Warshan D**, Sea-Yong K, Novotny A, Rasmussen U. Combined effects of elevated temperature and CO_2 alters epiphytic cyanobacterial community composition - consequences for nitrogen fixation activity and the host *Pleurozium schreberi*. *Manuscript*.

My contributions to the papers:

Paper I: Experimental design, cyanobacteria isolation from feathermosses, DNA isolation of feathermosses isolates, comparative genomic and phylogenetic analyses, writing.

Paper II: Performed experiments, nucleic-acid isolations, statistical and integrative analyses, writing.

Paper III: Nucleic-acid isolations, quantitative-PCR, statistical analyses, writing.

Paper IV: Experimental design, performed experiments, DNA library preparations, statistical analyses, writing.

Additional work completed during the PhD studies:

Hedberg N, Stenson I, Nitz-Pettersson M, **Warshan D**, Nguyen-Kim, Tedengren M, Kautsky N. Antibiotic use on Vietnamese fish and lobster sea cage farms and implications for the coral reef environment and human health. *Manuscript*.

Contents

Abbreviations

ANI	Average Nucleotide Identity
ATCC	American Type Culture Collection
BCAA	Branched-chained Amino Acid
GOE	Great Oxidation Event
GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamine Synthethase
H-NOX	Heme-nitric oxide/oxygen binding
HIF	Hormogonia Inducing Factor
HRF	Hormogonia Repressing Factor
Kbp	Kilo base pairs
LCA	Lowest Common Ancestor
Mb	Mega base
MCP	Methyl-accepting Chemotaxis Protein
NO	Nitric-Oxide
PCC	The Pasteur Culture collection of Cyanobacteria
PCR	Polymerase Chain Reaction
ppm	Parts per million
qPCR	Quantitative Polymerase Chain Reaction
RNAseq	Ribonucleic acid sequencing
SEM	Structural Equation Modelling
TCA	Tricarboxylic acid

Introduction

Evolutionary history and ecology of cyanobacteria

Cyanobacteria are the only organisms in which oxygenic photosynthesis has evolved and they are among the most ancient lineages that may have originated before 3,000 million years ago (Mya) (Schirrmeister et al., 2015). During the Precambrian (4,540–541 Mya), cyanobacteria were among the main drivers of life innovations and they indisputably impacted the early Earth's environments (Lyons et al., 2014). In the early Proterozoic (2,400–2,100 Mya), cyanobacterial photosynthetic activities generated a rapid oxygenation of Earth's atmosphere during an episode referred to as the 'Great Oxidation Event' (Castenholz et al., 2001; Bekker et al., 2004; Anbar et al., 2007; Fischer et al., 2016). This event increased oxygen (O_2) levels in the atmosphere that further allowed the emergence of complex aerobic life (Falkowski et al., 2005; Raymond and Segrè, 2006). The photosynthetic trait was acquired by other organisms, through endosymbiosis with cyanobacteria, resulting in the formation of chloroplasts (Sagan, 1967; Margulis, 1970). The endosymbiotic event between a cyanobacterium and a unicellular eukaryote that happened approximately 1,900 Mya (Sánchez-Baracaldo et al., 2017) is considered to be one of the most transformative events in the history of life. This event originated three groups of photosynthetic eukaryotes: red algae, green algae and plants, and glaucophyte algae (Archibald, 2009; Keeling, 2013), which in turn provided the endosymbionts in the subsequent evolution of lineages of photosynthetic protists and macroalgae (Gould et al., 2008; Archibald, 2015).

Cyanobacteria are a biochemically diverse clade of Gram-negative bacteria. Some cyanobacteria can fix atmospheric dinitrogen (N_2) and are considered among the main contributors to the global N cycle (Vitousek et al., 2002). For instance, the global input via N_2 fixation by the marine cyanobacterial genus *Trichodesmium* has been estimated to account for ~42% of the total global N_2-fixation (Berman-Frank et al., 2003). Biological N_2-fixation is the process by which atmospheric N_2, the most abundant gas in Earth's atmosphere, is chemically reduced to the equivalent of ammonia. In diazotrophs, organisms capable of N_2-fixation, the reaction is catalyzed by a multimeric enzyme complex called nitrogenase. Nitrogenase is a highly conserved enzyme and distributed in a diverse, but restricted, group of Bacteria

and Archea (Dos Santos et al., 2012; Boyd and Peters, 2013). The enzyme consists of a molybdenum-dependent ATP-hydrolyzing complex of two metalloproteins: a dinitrogenase heterotetramer ($\alpha_2\beta_2$ encoded by the *nifD* and *nifK* genes, respectively) that contains the active site for the reduction of N_2 and a dinitrogenase reductase homodimer (also referred as the iron (Fe) protein) that transfers high-energy electrons to dinitrogenase (Dos Santos et al., 2012). The nitrogenase is irreversibly inhibited by molecular oxygen and reactive oxygen species (Eady, 1996). Considering that cyanobacteria produce oxygen by photosynthesis specific adaptations for the combination of diazotrophy and oxygenic photosynthesis have evolved such as separation in time or space (Stal et al., 2010). For instance, some cyanobacteria can differentiate N_2-fixing cells called heterocysts, which are spatially separated from photosynthetic (vegetative) cells and possess an additional thick envelope to protect the nitrogenase enzyme from oxygen deactivation (Kumar et al., 2010).

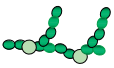
Cyanobacteria have a high degree of diversity in their morphological features compared to other prokaryotes. They can be unicellular, colonial, or filamentous (Table 1). A combination of phylogenetic and morphological characters is used to divide the group into five subsections: (I) the Chroococcales, unicellular coccoids that divide by binary fission; (II) the Pleurocapsales which are unicellular and go through multiple fission to produce small cells used for dispersion, called baeocytes; (III) the Oscillatoriales, forming filaments with only vegetative cells that divide in one plan; (IV) the Nostocales, filamentous but their vegetative cells can differentiate into heterocysts, motile filaments called hormogonia or spore-like resting cells called akinetes, that survive environmental stresses. The cells divide in one plan; (V) the Stigonematales, filamentous branching patterns where vegetative cells can differentiate into heterocysts, hormogonia or akinetes. The cells divide in multiple plans (Table1) (Castenholz et al., 2001). Nevertheless, this traditional taxonomic classification of cyanobacteria is still debated (Dvořák et al., 2017).

The geologic record provides insight into the early life of cyanobacteria. Molecular microfossils considered to be produced by cyanobacteria (2-methylhopanoids) have been dated at ca. 2,700 Mya (Brocks et al., 1999). Microfossils harbouring common morphological attributes with extant cyanobacteria belonging to the subsections I–III were dated at ca. 1,900 Mya (Hofmann, 1976). Geological records contain many fossils of akinetes that have been identified in several sites ranging between 1,650–1,200 Mya (Sergeev, 2009), and the oldest known fossil of an akinete was preserved in a chert dated ca. 2,100 Mya (Tomitani et al., 2006). Heterocysts has been proposed to be an adaptation for fixing N_2 when oxygen level in the atmosphere reached deleterious level for nitrogenase ca. 2,450–2,320 Mya (Tomitani et al., 2006). Phylogenetic analysis coupled with the previous geological evidence suggest that the monophyletic group of cyanobacteria capable of cell

differentiation (subsections IV and V) diverged once between 2,450 and 2,100 Mya (Tomitani et al., 2006).

During their long evolutionary history, cyanobacteria established in nearly all habitats that have access to light, ranging from the arctic tundra to the desert, warm springs and all marine and freshwater habitats (Potts and Whitton, 2000). Based on their photoautotrophic nature cyanobacteria can live as free living organism but can also form symbioses with taxonomically diverse hosts, such as dinoflagellates, ciliates, radiolarian (Foster et al., 2006), microalgae (Zehr et al., 2016), diatoms (Janson, 2002; Carpenter and Foster, 2002; Foster et al., 2011), sessile invertebrates like sponges, ascidians and cnidarians (Carpenter and Foster, 2002; Lesser et al., 2004; Larkum and Kühl, 2005), echiuroid worms (Raven, 2002), fungi (Rikkinen, 2017), and plants (Rai et al., 2000). In symbiosis, cyanobacteria can provide a non-photosynthetic host with a source of reduced carbon (C) coming from their photosynthetic activity. This is, for example, the case in the symbiosis between cyanobacteria and marine invertebrates and in terrestrial and marine bipartite cyanolichens (involving a fungus and a cyanobacterium) (Usher et al., 2007). In symbiosis with plants, tripartite cyanolichens (fungus, algae and cyanobacterium partners), microalgae and marine diatoms, the cyanobacteria provide the host with a source of reduced N such as ammonium coming from their N_2-fixation activity (Usher et al., 2007; Meeks, 2009; Foster et al., 2011; Bay et al., 2013; Zehr et al., 2016).

Table 1. Cyanobacteria morphological classification.

	Subsection	Order	Type
	Subsection I	Chroococcales	Unicellular coccoids, divide by binary fission.
	Subsection II	Pleurocapsales	Unicellular or colonial, divide by multiple fission.
	Subsection III	Oscillatoriales	Filamentous with only vegetative cells. Divide solely in one plane.
	Subsection IV	Nostocales	Filamentous with vegetative cells that can differentiate into heterocyst, hormogonia and akinetes. Forming false-branching or no branching, and divide solely in one plane.
	Subsection V	Stigonematales	Filamentous with vegetative cells that can differentiate into heterocyst, hormogonia and akinetes. Forming true-branches i.e. cell division in more than one plane.

Information from (Castenholz et al., 2001).

Terrestrial symbiosis with cyanobacteria

Terrestrial symbioses with cyanobacteria are involving fungi (lichens and *Geosiphon*) and plants from many major lineages of non-vascular and vascular land plants (Rai et al., 2000). Symbiotic cyanobacteria (cyanobionts) can have different degrees of integration or 'intimacy' with their host. They may be extracellular, living epiphytically or in specialised compartments, but can also be intracellularly localized e.g. in stem glands of the angiosperm of the genus *Gunnera* or in the bladders of the fungus *Geosiphon pyriformis* (Kiitz.) von Wettstein (Figure 1) (Rikkinen, 2017). Almost all cyanobacterial-plant symbioses are formed when the host is N limited and are facultative, i.e. the cyanobacteria and the plant can live independently or enter a symbiosis. The only known exception is the obligate symbiosis with the water fern of the genus *Azolla* (Rai et al., 2000). The host plants are all ancient lineages, which implies a potential long history of co-evolution between plants and cyanobacteria (Usher et al., 2007).

Bryophytes in symbiosis with cyanobacteria

Bryophytes (mosses, liverworts and hornworts) are non-vascular plants and are generally recognized as the oldest living land plants, having evolved ca. 470 Mya (Edwards et al., 2014; Brown et al., 2015). When in symbiosis with bryophytes the cyanobacteria have an extracellular lifestyle; they are commonly found as epiphytes on the moss gametophyte or as endophytes inside cavities of liverworts and hornworts (Rikkinen, 2017) (Figure 1). The mosses (Bryophyta) are the largest group of bryophytes comprising around 10,000 species. Mosses in symbiosis with N_2-fixing cyanobacteria are distributed in all latitudes ranging from arctic tundra and temperate forests to tropical ecosystems and Antarctica. In some of these habitats where atmospheric N deposition is low, the two partners play a pivotal role for N and C cycles; for instance in boreal forest ecosystems, the symbiosis with feathermosses is responsible for the main biological input of N into this ecosystem (DeLuca et al., 2002).

In the boreal forest, the most common feathermosses species are *Pleurozium schreberi* (Brid.) Mitt., *Ptilium crista-castrensis* (Hedw.) De Not. and *Hylocomium splendens* (Hedw.) Schimp., which can be found associated with a high diversity of cyanobacteria (Figure 1) (Gentili et al., 2005; Houle et al., 2006; Ininbergs et al., 2011). In the subarctic and arctic tundra, N_2-fixing cyanobacteria in symbiosis with the peat moss *Sphagnum* are a main source of 'new' N for these ecosystems, with N_2-fixation rates exceeding atmospheric N deposition (Zielke et al., 2005; Stewart et al., 2011). In symbiosis with *Sphagnum* the cyanobacteria are often present epiphytically but are also found in dead and water-filled hyaline cells present in the leaves and

stems (Solheim and Zielke, 2002). Recent studies reported the occurrence of these symbioses with other moss species, such as in North-American temperate forest with the mosses *Thuidium delicatulum* (Hedw.) Schimp. and *Fissidens taxifolius* Hedw. (Deane-Coe and Sparks, 2015). In tropical regions, cyanobacteria in symbiosis with arboreal mosses were shown to have the largest potential N input in two Costa Rican forests (Cusack et al., 2009). In Antarctica at Terra del Fuego, mosses of the genera *Andreaea*, *Acroshima*, *Ditrichum*, *Dicranoloma*, and *Racomitrium* are forming symbioses with cyanobacteria (Arróniz-Crespo et al., 2014). Those recent findings suggest that moss-cyanobacteria symbioses might be more wide-spread than previously thought, and exist even in environments where atmospheric N deposition was considered to be extremely high (Deane-Coe and Sparks, 2015).

Among the 13 genera of hornwort (Anthocerotophyta) known, all can form a symbiosis with cyanobacteria (Renzaglia et al., 2007). By contrast only four out of 340 genera of liverworts can form a symbiosis with cyanobacteria (Adams and Duggan, 2008). Among the four genera of liverwort (Marchantiophyta), *Blasia pusilla* L. and *Cavicularia densa* Steph. (Blasiales) are the only liverworts that form morphologically well-defined symbioses, where the cyanobacteria are located inside cavities in the thallus, called auricles (Figure 1) (Adams and Duggan, 2008). The genera *Marchanthia* and *Porella* have epiphytic cyanobacteria. It is important to notice that the thallus cavities hosting the cyanobacteria of liverworts (auricles) and hornworts (mucilage cavities), are not specifically produced as response to the presence of the cyanobiont (Renzaglia et al., 2000).

Ferns in symbiosis with cyanobacteria

The water fern genus *Azolla* (Salviniales, Pteridophyta) is the only fern found in symbiosis with cyanobacteria, and this is also the only obligate cyanobacterial-plant symbiosis described so far (Adams et al., 2013). The origin of the genus *Azolla* was estimated at ca. 90 Mya (Metzgar et al., 2007). This genus contains seven extant species (Pereira et al., 2011), where several of them are widely distributed in tropical and warm temperate regions. The *Azolla* symbiosis is of considerable economic importance, since it can be used as a N-rich biofertilizer especially in rice paddies (Brouwer et al., 2014). The cyanobacteria are located extracellularly in dorsal leaf cavities (Figure 1) that can contain between 2,000–5,000 cyanobacteria cells, immobilized within an exopolysaccharide-rich mucilage (Lechno-Yossef and Nierzwicki-Bauer, 2002). The cyanobacterium of *Azolla* is vertically transmitted to new generations of the fern by the female reproductive structure (megasporocarp). During colonization of the megasporocarp, hormogonia enter through pores at the top of the indusium and then differentiate into akinetes, the resting stage of

heterocystous filamentous cyanobacteria (Zheng et al., 2009). After fertilisation of the megasporocarp and embryogenesis, the akinetes germinate to form the cyanobacteria community associated within the newly formed embryonic leaf (Zheng et al., 2009).

Gymnosperms in symbiosis with cyanobacteria

The cycad-cyanobacteria symbiosis is the only known example of a plant root-cyanobacteria symbiosis and also the only gymnosperm-cyanobacteria symbiosis. Cycads are an ancient lineage of evergreen, palm-like gymnosperms. The origin of the lineage is dated between 265–290 Mya (Brenner et al., 2003; Condamine et al., 2015). The extant cycads include approximately 300 species that are classified in 12 genera, distributed in tropical and subtropical regions of the Americas, Africa, Southeast Asia, and Australia (Wang and Ran, 2014). Cycads can vary in height from 20 cm to almost 20 m at maturity. It has been proposed that dinosaurs were key dispersers of cycad seeds during the Mesozoic era (252–66 Mya) because temporal variation in cycad diversity and abundance has been correlated to changes in occurrences of herbivorous dinosaurs (Salgado et al., 2017). The cyanobiont is hosted in specialized coralloid roots (Figure 1). The colonization of the coralloid root is still not well-characterized but it is thought to occur through apical lenticels and/or injured epidermal cells (Lindblad, 2008). After entering the coralloid root, the cyanobacteria establish a zone between the inner and outer cortex, where they fix N_2 and transfer the products containing reduced N to the host in exchange for C sources (Lindblad, 2008).

Angiosperms in symbiosis with cyanobacteria

The extant genus *Gunnera* is composed of 30–40 species; they are all perennial, with one exception, *G. herteri*, and they vary considerably in size from a few centimetres up to a few meters tall (Wanntorp et al., 2001; Wanntorp and Wanntorp, 2003; Rutishauser et al., 2004). *Gunnera* species are found in South America, Africa, and the Australasian region, but endemic species can be found also in Hawaii, and *G. mexicana* is found in southern Mexico (Wanntorp and Wanntorp, 2003). All *Gunnera* species can form symbiosis with cyanobacteria and this is the only known symbiosis between an angiosperm and cyanobacteria. The divergence time of the order Gunnerales from the core eudicot lineage was estimated at ca. 115 Mya (Vekemans et al., 2012), which makes this symbiosis relatively recent compared to the other plant-cyanobacteria symbioses. The cyanobionts are localized intracellularly in the stem cortex of the plant, where they colonize glands that are formed below the leaf petioles (Bergman, 2002; Osborne and Sprent, 2002). The gland secretes mucilage which induces hormogonium differentiation in the

cyanobacterium (Rasmussen et al., 1994, 1996). The mucilage attracts hormogonia between the gland's papillae and further inside the gland where the cyanobacteria enter into the plant cells (Rasmussen et al., 1994, 1996; Uheda and Silvester, 2001). Within the plant cells, the cyanobacteria are surrounded by the host cell plasmalemma and the membrane acts as the interface through which the exchange of metabolites takes place (Bergman, 2002). Gland production in *Gunnera* does not occur as a response to the presence of symbiotic cyanobacteria. The trigger of their development seems to be N limitation, therefore the symbiotic event is considered to be controlled by the host (Adams et al., 2013).

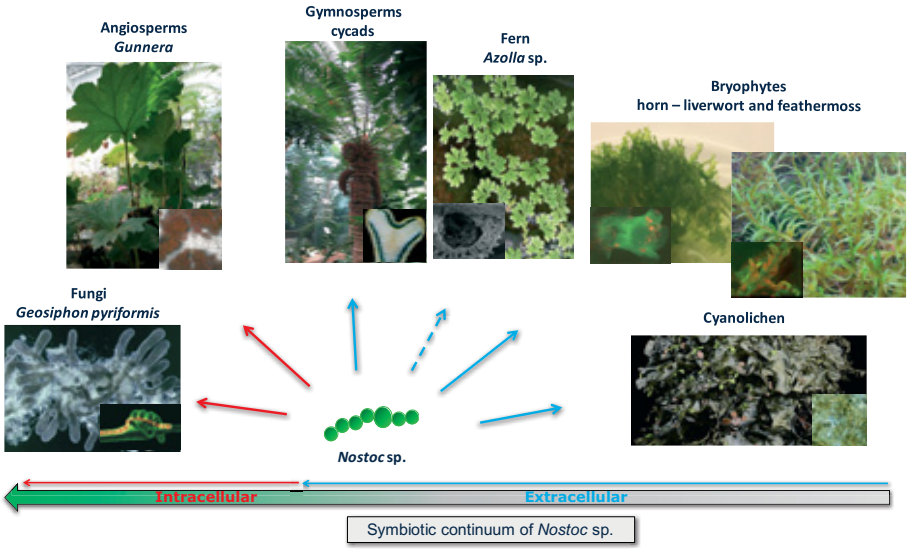


Figure 1: Symbiotic continuum of terrestrial *Nostoc.* The cyanobacteria in facultative symbiosis with plants can live extracellularly (blue line) as epiphytes of feathermoss, in cavities of the thallus of hornworts/liverworts, in collaroid roots of cycads, and intracellularly (red line) in *Gunnera* sp. stem glands. The symbiosis with the fern *Azolla* is obligate for both the plant and the cyanobacterium (dotted line). Terrestrial *Nostoc* sp. can enter in symbiosis with fungi, extracellularly with bi- tripartite lichen and intracellularly with *Geosiphon pyriformis*. Photos of cyanolichen and *Geosiphon pyriformis* are from Adams et al. (2013) and Rikkinen (2017) with permission, additional photos from Ulla Rasmussen and Denis Warshan.

Cyanobacteria in symbiosis with plants

All plant cyanobionts have characteristics commonly shared with other cyanobacteria, such as the ability to differentiate both heterocysts for diazotrophy and hormogonia for motility. Although many cyanobacteria have these two characteristics, only a few genera can form a symbiosis with plants, meaning

that other prerequisites are of importance for becoming a cyanobiont. For instance, a cyanobiont needs to be capable of chemotaxis to move towards the host plant and adaptable to the conditions offered by the host. This encompasses metabolic plasticity to shifts from photo-autotrophy to heterotrophic C metabolism, and to be able to exchange metabolites with the host (Adams et al., 2013). The few genera of cyanobionts able to fulfill these prerequisites belong to the order Nostocales and Stigonematales (subsections IV and V; Castenholz et al., 2001). *Nostoc* is the main genus found in symbiosis with plants from all lineages; *Nostoc* strains possess high degree of phenotypic plasticity to adapt to new environments (Figure 2).

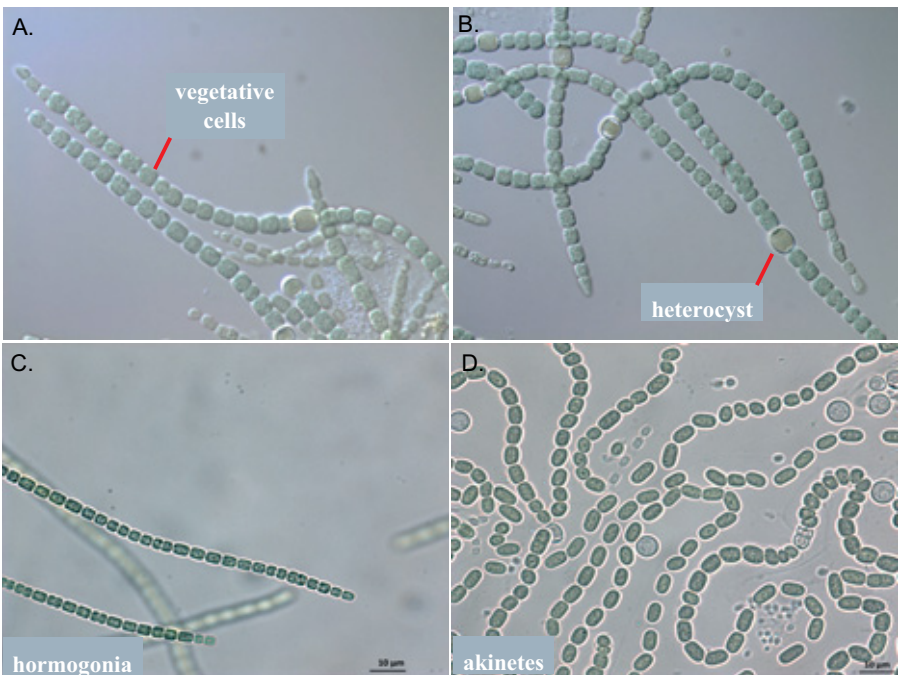


Figure 2: Morphological plasticity of *Nostoc.* The filament is formed by (A) vegetative cells that can differentiate into (B) N_2-fixing cells called heterocyst, (C) hormogonia, motile filament and (D) akinetes, spore-like resting cells. All photos were taken at magnification x100. Photos Ulla Rasmussen and Denis Warshan.

To date, genomic information for symbiotic cyanobacteria were only available for the facultative symbiont *Nostoc punctiforme* ATCC 29133, isolated from the cycad *Macrozamia* sp. (identical to *N. punctiforme* PCC 73102), and *Nostoc azollae,* the obligate partner of *Azolla* sp. (Meeks et al., 2001; Ran et al., 2010). Interestingly, symbiosis reconstruction experiments with *N. punctiforme* showed that this strain has a broad host-range and can establish all facultative symbiotic relationships known between plants and cyanobacteria,

extending from symbiosis with liverworts, hornworts to the intracellular symbiosis with *Gunnera* sp. (Campbell and Meeks, 1989; Johansson and Bergman, 1994). Moreover, phylogenetic inference of cyanobacteria associated with liverworts/horworts and *Gunnera* sp. showed that most of the cyanobacteria are closely related to *N. punctiforme* (Svenning et al., 2005), suggesting that the capability to have a broad host-range might be more widespread than reported. Plant symbiotic *Nostoc* strains can also form a symbiosis with a fungus, for instance *N. punctiforme* forms an intracellular symbiosis with *Geosiphon pyriformis*, a glomeromycotan fungus (Schüßler et al., 1994; Schüßler, 2012). Moreover, symbiotic *Nostoc* strains of many cyanolichens, including both bi- and tripartite species, have been identified as *N. punctiforme*. Genotyping using different phylogenetic markers (partial 16S rDNA, *rbcLX,* and *trnL* sequences) of several cyanobionts indicate a close relatedness between *Nostoc* genotypes of lichen species with those from plant and/or environmental samples (O'Brien et al., 2005; Stenroos et al., 2006; Myllys et al., 2007; Papaefthimiou et al., 2008a). This indicates that host specialization among symbiotic *Nostoc* genotypes might be low, and that co-evolution with only one host partner is rare among cyanobionts (Rikkinen, 2017). As cyanolichens are usually found together with other *Nostoc* sp. hosts, such as mosses, hornworts and liverworts it can be hypothesized that a common evolutionary history could exist among fungi, lichens, vascular plants, bryophytes and symbiotic *Nostoc* (Rikkinen, 2017).

Cyanobacteria in symbiosis with bryophytes

The cyanobacterial community living in symbiosis with the feathermosses *H. splendens* and *P. schreberi* is the most diverse among all plant-cyanobacteria symbioses, which might be due to its epiphytic nature (Figure 3) (Ininbergs et al., 2011). These communities have been characterized primarily through morphological observations and culture-dependent studies, and are composed of species belonging to the genera *Nostoc* (subsection IV), *Calothrix* (IV), *Fischerella* (V), and *Stigonema* (V) (Gentili et al., 2005; Houle et al., 2006; Zackrisson et al., 2009). In the study by Ininbergs et al. (2011), a partial fragment of the *nifH* gene (encoding the iron-protein component of the nitrogenase complex) was used as a molecular marker for investigating the diversity of the cyanobacterial community inhabiting *P. schreberi* and *H. splendens* (Figure 3). They showed that the cyanobacterial community is host-specific and composed of five different phylogenetic clusters that were represented by *nifH* phylotypes belonging to the genera mentioned above. Those *nifH* clusters are two '*Nostoc*' clusters (i.e. '*Nostoc* cluster I' and '*Nostoc* cluster II'), a '*Stigonema* cluster', a '*nifH*2 cluster' represented by the genera *Calothrix* and *Fischerella*, and a 'mixed' cluster characterized by several Nostocales genera (Figure 3).

Several studies have investigated the genetic diversity of cyanobacterial communities in symbiosis with the moss *Sphagnum* and the liverwort and hornwort symbioses. For *Sphagnum*, *nifH* amplicon sequencing of the associated communities led to the identification of sequences affiliated with section IV cyanobacterial genera *Anabaena*, *Tolypothrix* and *Aphanizomenon* (≥95% similarity) and for the liverworts and hornworts, based on the $tRNA^{Leu}$(UAA) intron, the cyanobacteria hosted belong to the genus *Nostoc* (Costa et al., 2001; Rikkinen and Virtanen, 2008; Bragina et al., 2012; Rikkinen, 2013). The thallus of liverworts and hornworts were found to be inhabited by different *Nostoc* strain but only one strain in each individual cavity (Costa et al., 2001; West and Adams, 1997).

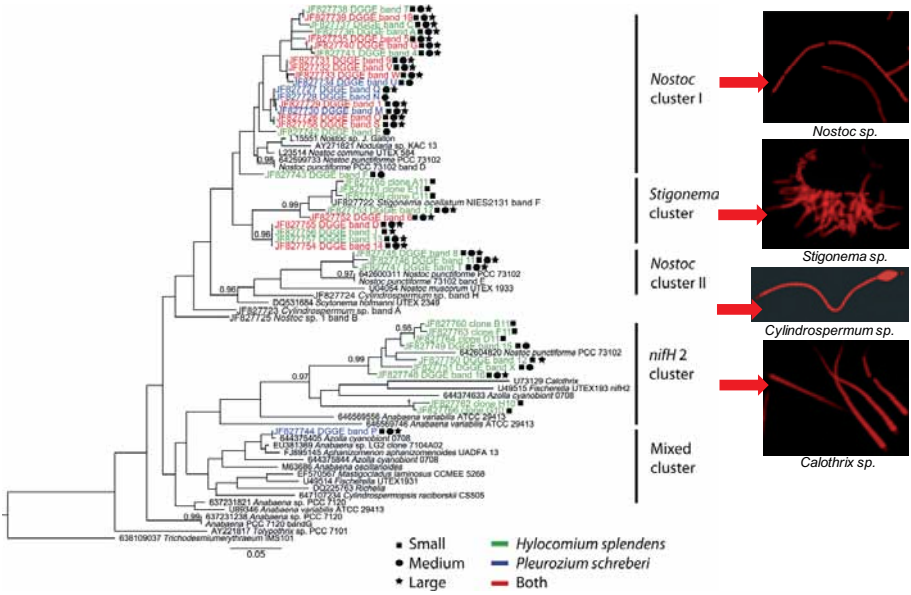


Figure 3: Maximum-likelihood phylogenetic tree from alignments of partial *nifH* genes amplified from *H. splendens* and *P. schreberi* (color coded). Moss were sampled in 30 lake islands varying in size (small, medium, and large) located in the Reivo forest, northern Sweden. The cyanobacteria community is represented by four main clusters. A representative of each cluster was imaged using epifluorescence microscopy to visualize cyanobacterial phycobilisome-mediated fluorescence (bright red). Adapted from Ininbergs et al. (2001) with permission. Additional photos from Ulla Rasmussen.

The cyanobionts of Azolla sp.

In the water fern *Azolla*, the cyanobionts are living endophytically in leaf cavities, which are colonized by a 'primary' cyanobacterium, named *Nostoc azollae*, which is dominating the community and is unable to grow without its

host plant. The cyanobacterial community in the *Azolla* leaf cavity also contains 'secondary' facultative symbiotic strains which were affiliated with the genus *Anabaena* (Papaefthimiou et al., 2008b; Sood et al., 2008). The taxonomy of the primary and secondary cyanobacterial symbionts in the actual Nostocales classification is still debated, and the genus of these cyanobacteria still needs to be investigated (Pereira and Vasconcelos, 2014).

Cyanobacteria in symbiosis with cycads

The symbiotic cyanobacteria of cycads are usually *Nostoc* strains, but also *Calothrix* strains have been found (Gehringer et al., 2010). There seems to be little host specificity between cycad species and their cyanobionts, since one plant can host different *Nostoc* strains, and even a single coralloid root can be inhabited by different strains (Costa et al., 1999; Zheng et al., 2002; Gehringer et al., 2010; Thajuddin et al., 2010; Yamada et al., 2012). The *Nostoc* strains in symbiosis were found to be phylogenetically distinct from those found in liverworts and hornworts and more closely related to some free-living *Nostoc* strains (Rikkinen and Virtanen, 2008; Gehringer et al., 2010).

Cyanobacteria in symbiosis with Gunnera

Molecular studies of the cyanobacteria in symbiosis with *Gunnera* show that the cyanobionts exclusively belong to the genus *Nostoc* and are closely related to some symbiotic and free-living representatives of the genus (Rasmussen and Svenning, 2001; Svenning et al., 2005; Papaefthimiou et al., 2008a). Phylogeny of *Nostoc* strains in symbiosis with *Gunnera* showed that they can also form a clade separate from other plant-associated strains (Svenning et al., 2005). Those other symbiotic *Nostoc* strains are intermixed with free-living representatives, suggesting that the symbiotic capability arose several times during evolution (Svenning et al., 2005). Different *Nostoc* strains may be found in a single *Gunnera* plant (Nilsson et al., 2000), but generally, one plant hosts one strain (Guevara et al., 2002). The *Nostoc* cyanobionts of *Gunnera* sp. are closely related to those found in cyanolichens and bryophytes (Rikkinen and Virtanen, 2008). *Nostoc* isolates from cycads and bryophytes also readily invade *Gunnera* cells and *vice versa*, which is pointing towards that cyanobionts have a low-host specificity (Adams et al., 2013).

Mechanisms and molecular pathways involved in the establishment and maintenance of cyanobacteria-plant symbioses

Current knowledge on colonization steps and maintenance of cyanobacteria-plant symbioses is primarily based on studies of symbioses with the liverwort *Blasia,* the hornworts *Anthoceros* and *Phaleceros*, and with the angiosperm

Gunnera (Adams and Duggan, 2012). During the establishment of these symbioses, there are two phases of interaction, an early phase, which includes chemical signaling between partners, followed by a later phase where the cyanobacteria are physically associated with the host (Figure 4).

In the early phase, the cyanobacteria differentiate from vegetative filaments into a transient motile stage termed hormogonia (Figure 4). The differentiation can be induced by a variety of environmental stimuli and host-produced chemical signals of unknown structure known as Hormogonia Inducing Factor, HIF (Rasmussen et al., 1994; Meeks and Elhai, 2002; Adams and Duggan, 2008). Cyanobacterial genes responding to plant HIF have been identified, and many appear to be involved in signal transduction and transcriptional activation (Campbell et al., 2007; Duggan et al., 2013; Risser and Meeks, 2013; Risser et al., 2014; Campbell et al., 2015). A recent study has identified *che*-family genes as important for both motility and symbiotic competence (Duggan et al., 2013), while genes involved in phototaxis, chemotaxis, and motility (*ptx*, *hmp*, *hps*, and *pil* genes) are thought to be implicated in the colonization process (Campbell et al., 2008; Risser and Meeks, 2013; Risser et al., 2014). According to the only study addressing feathermoss colonization, the feathermoss secretes HIF and possibly chemo-attractants, to recruit and direct the hormogonia towards the host (Bay et al., 2013). Results of the previous studies suggest that gene expression patterns of the feathermoss and cyanobacteria will change dramatically during recruitment and subsequent association. For example, feathermoss typically become more permissive to bacterial growth during association with cyanobacteria (Rousk et al., 2013). While the mechanism of this phenomenon is unknown, it may be related to a reduced production of antimicrobial cationic peptides (Zasloff, 2002; Skripnikov et al., 2011) or oxylipin molecules (Matsui, 2006; Croisier et al., 2010) synthesized by the moss.

The initial signaling and motility stage is followed by a physical interaction phase between host and cyanobacterium (Figure 4). In symbiosis with hosts such as *Gunnera*, *Blasia* and *Anthoceros*, the cyanobacteria exhibit shared morphological and metabolic rewiring to adapt to the new environment offered by the plant (Adams and Duggan, 2012). Further, establishment of a functional symbiosis culminates in the differentiation of vegetative cyanobacterial cells into N_2-fixing cells, heterocysts, at a very high frequency, which is also associated with the regulation of a unique set of genes (Golden and Yoon, 2003; Meeks, 2006; Muro-Pastor and Hess, 2012). Specific changes include repression of hormogonia development regulated by the *hrm* locus, increases in heterocyst frequency controlled by the *hetR* gene and N_2-fixation activity by *nif* genes, repression of ammonium assimilation, i.e. of GS-GOGAT activity (glutamine synthethase *glnA* and glutamate synthase *gltB*), and reduced photosynthetic CO_2 fixation (Adams and Duggan, 2012). As the

biochemistry associated with N_2-fixation requires a lot of energy and the photosynthetic activity by the cyanobionts is repressed (due to reduced or no light), it is believed that the plants supply sugar as an energy source to their partner (Steinberg and Meeks, 1991; Meeks, 2009). These changes imply that the cyanobiont shifts from photo-autotrophy to heterotrophic carbon metabolism, with organic C provided by the host to sustain N_2-fixation (Meeks and Elhai, 2002). The carbohydrate exported from the host is not identified for Bryophytes but in *Gunnera* it was suggested to be glucose and/or fructose (Khamar et al., 2010). In exchange, the cyanobacteria release 80–90% of their fixed N, mainly as ammonia, as shown in the case of *Gunnera*, liverworts and hornworts symbioses (Meeks, 2009). No studies have been investigating if the molecular pathways described above are also involved in the establishment and maintenance of the feathermoss-cyanobacteria symbiosis.

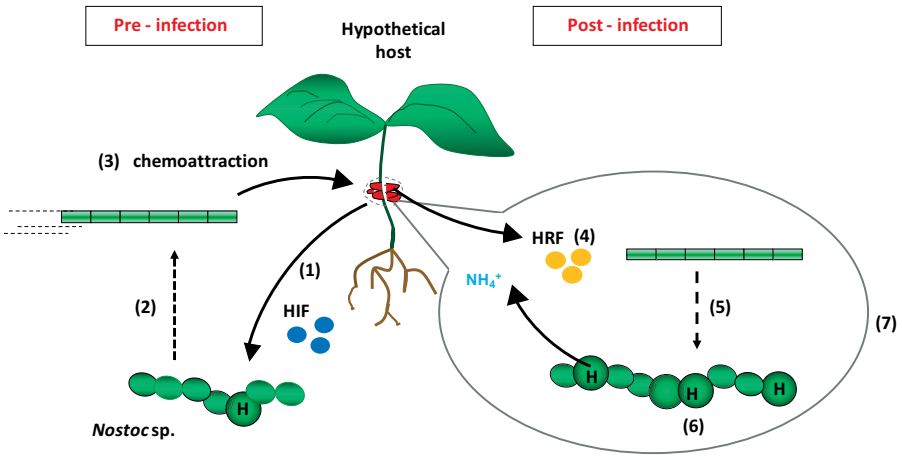


Figure 4: Schematic representation of symbiotic events involved in the establishment and maintenance of a cyanobacterium and a host plant. (1) Plant produces a hormogonia inducing factor, HIF (blue) (2) HIF induces cyanobacterial hormogonia differentiation (motile form) (3) Hormogonia are subsequently attracted by chemotaxis towards their symbiotic partner (4) Plant produces a hormogonia repressing factor, HRF (yellow) (5) Cyanobacteria turn into vegetative cells (6) Heterocysts (H) frequency is increased (7) N is transferred to the plant usually as ammonium (NH_4^+). Adapted from Ulla Rasmussen.

Feathermosses-cyanobacteria symbiosis as contributor of boreal forests productivity

Many terrestrial surfaces, such as soils, rocks and plants, are covered by cryptogamic communities comprising different proportion of free-living cyanobacteria and/or several eukaryotes - algae, fungi, lichens and bryophytes, capable of fixing N_2 and CO_2. The N input by these covers accounts for nearly

half of the biological N_2-fixation on land (Elbert et al., 2012), turning cryptogamic covers into major players of the global biogeochemical cycles of C and N.

Among the cryptogams, cyanobacteria in symbiosis with plants play a pivotal role in the productivity of their respective ecosystems. Establishing a symbiosis with an N_2-fixer can be of crucial importance for the hosts to grow in habitats with low soil N availability (Werner et al., 2014). For instance, N_2-fixation by cyanobacteria living in symbiosis with *P. schreberi* and *H. splendens* and *Sphagnum fuscum* serve as the primary input of nitrogen into boreal forests and sub-arctic ecosystems, where they can account for 50% of total N input (DeLuca et al., 2002, 2008; Turetsky et al., 2012; Rousk et al., 2015). *P. schreberi* and *H. splendens* are pleurocarpous mosses; i.e. their sporophytes are developing on a side branch rather than on the main stem. They have a feather-like foliage (hence the name feathermoss), and are ubiquitous in boreal forests, where they can cover more than 80% of the forest floor (Figure 5) (DeLuca et al., 2002; Zackrisson et al., 2009). The net productivity of the forest understory composed of dwarf shrubs and these two mosses can in some areas represent a large fraction of the total net productivity, and may exceed that of trees (Lindo et al., 2013; Wardle et al., 2012). The moss cover represents an important compartment in boreal forests, contributing to N and C dynamics in these ecosystems (Lindo and Gonzalez, 2010; Lindo et al., 2013). N input by cyanobacteria in symbiosis with feathermosses is on par with the atmospheric N deposition, the other main source of N input, in boreal ecosystems (Gundale et al., 2011). High-resolution secondary ion mass spectrometry (SIMS) showed that fixed N was transferred from the cyanobacteria to the moss host and accumulated in the moss tissue (Bay et al., 2013). N is presumably released in the ecosystem through three pathways; after events like drying-rewetting and fire disturbance, by mineralization processes and by interaction with mycorrhiza (Lindo et al., 2013). Long-term boreal fire chronosequence showed that in old forests where nutrient mineralization and availability are low, vascular plants and feathermosses $\delta^{15}N$ isotopic signature converged near to the value of newly fixed N by cyanobacteria (Hyodo and Wardle, 2009). This suggests that the vascular plants can directly acquire recently assimilated N from feathermosses, for instance through mycorrhizal association or drying-rewetting events. Even though, feathermosses retain a majority of the newly fixed N, at intermediate temporal scales (years to decades) N is released in the soil as bryophyte litter, and may become available to vascular plants through decomposition processes (Hyodo and Wardle, 2009; Gavazov et al., 2010). Over long time scales (decades to century), fire disturbances can serve as a third main pathway through which N is transferred from feathermosses to vascular plants (Lindo et al., 2013). Despite the contribution of feathermosses and N_2-fixing cyanobacteria to the boreal forests N

budget, this ecosystem is N limited. Many studies have shown that N availability is a limiting factor for primary productivity in the boreal forest ecosystem (Hungate et al., 2003; Reich et al., 2006; Gerber et al., 2010)

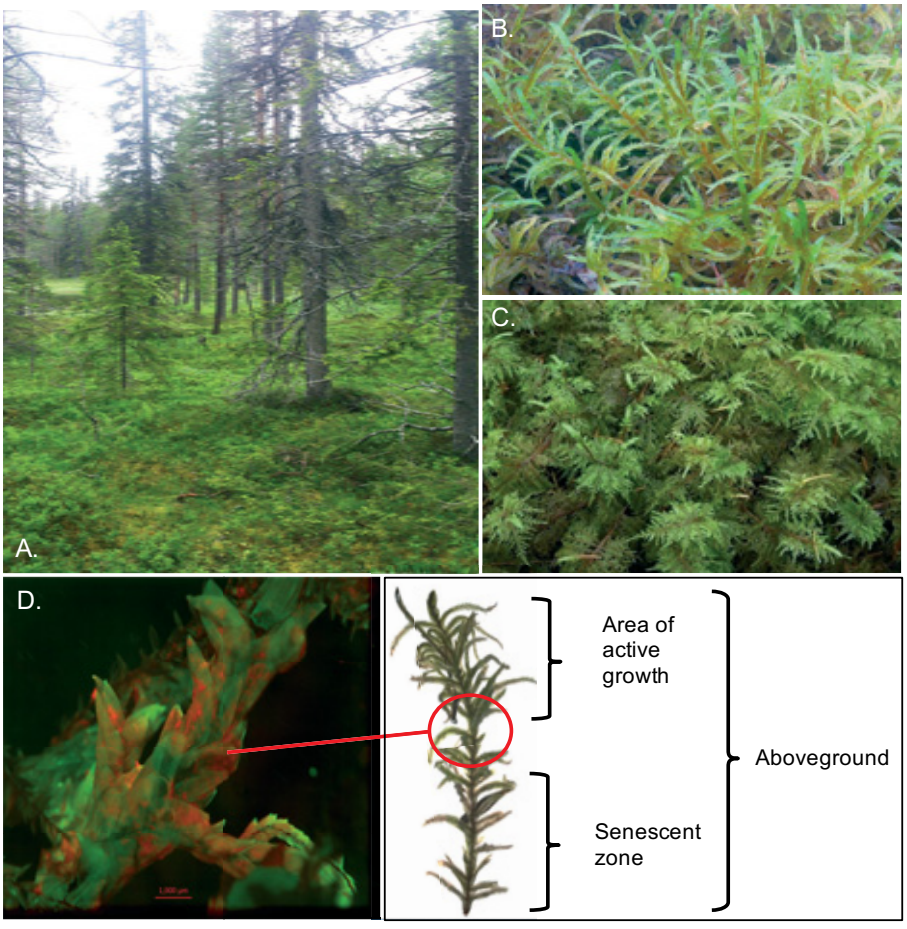


Figure 5: Boreal forest at Ruttjeheden, Reivo forest, northern Sweden (A) with the forest floor covered in *P. schreberi* (B) and *H. splendens* (C). *P. schreberi* associated with cyanobacteria (D) imaged under epifluorescence microscopy to visualize cyanobacterial phycobilisome-mediated fluorescence (red), and localization of the epiphytic cyanobacteria on the moss gametophyte (red circle). Photos by Ulla Rasmussen and Denis Warshan.

Abiotic and biotic factors influencing the N_2-fixation activity by feather-mosses-symbiotic cyanobacteria

Abiotic regulators of cyanobacterial N_2-fixation activity

N_2-fixation activities in the boreal forest ecosystems often increase with the age of the forest (DeLuca et al., 2008) and increases over time after disturbances, such as fire events (Lagerström et al., 2007). Furthermore, different species of cyanobacteria perform N_2-fixation at different rates, depending on temperature (Gentili et al., 2005; Sorensen and Michelsen, 2011; Lindo and Griffith, 2017), water availability (Gundale et al., 2009, 2012a), and light intensity (Gundale et al., 2012b). The interactive effect between light intensity and temperature has also to be considered, with a positive effect of high light intensity (517 μmol m^{-2} s^{-1}) on N_2-fixation rates at 16°C but possible damage to cyanobacteria cells at 30°C (Gundale et al., 2012b).

In the boreal forest, the feathermoss carpets experience intense drying and rewetting events: the moss becomes dry during long warm days and rewets during rainfall events (Jackson et al., 2011). Mosses were shown to be resilient to extreme drying events and rapidly recover their normal metabolism and growth after rewetting (Proctor, 2001). The recovery of photosynthesis can happen within minutes after rehydration, with some moss species starting to fix CO_2 immediately after rehydration (Proctor, 2001; Proctor et al., 2007). However, N_2-fixation by the symbiotic cyanobacteria was shown to be inhibited by drying events and rapid temperature variations. As an example, the recovery of N_2-fixation activities after rewetting of dried moss takes ca. five days to reach the previous measured values (Ackermann et al., 2012; Gundale et al., 2012b; Rousk et al., 2014a).

Availability of nutrients on the feathermoss also controls N_2-fixation activities, which increase upon addition of molybdenum (Mo), and decrease with phosphorus (P) limitation (Rousk et al., 2016). The chemistry of N_2 reduction is constrained by Mo since a tetrameric Mo-iron (Fe) protein subunit is part of the nitrogenase enzyme complex (Eady, 1996). Therefore, Mo but also Fe limitations drastically reduce N_2-fixation activity in terrestrial systems (Zhang et al., 2016). Three iso-enzymes of nitrogenase have been documented so far, the Mo-Fe nitrogenase and two alternatives, the vanadium (V)-Fe dependent nitrogenase and the Fe-only nitrogenase (Eady, 1996; Reed et al., 2011). Recently, it was suggested that the cyanobiont of the cyanolichen *Peltigera aphthosa* found in boreal forests can use the V alternative nitrogenase to fix N when V is present and Mo is limited, implying that our understanding of micronutrient constraints on N_2-fixation should also integrate V availability (Zhang et al., 2016; Darnajoux et al., 2017).

Several studies have shown that both N_2-fixation and feathermoss biomass decrease with increased N deposition (Zackrisson et al., 2004; DeLuca et al., 2008; Gundale et al., 2011; Ackermann et al., 2012; Gundale et al., 2013; Rousk and Michelsen, 2016). N additions on par with higher levels of atmospheric N deposition (12.5 kg N ha^{-1} yr^{-1}) reduce the feathermoss biomass by 29%, and inhibit the N_2-fixation rate per area unit by 58% (Gundale et al., 2013). However, studies showed that N fertilization with up to 10 kg N ha^{-1}

(Rousk et al., 2014b) did not systematically inhibit N_2-fixation in *P. schreberi* and was even promoting it at lower fertilization levels (5 kg N ha^{-1} yr^{-1}) (Rousk and Michelsen, 2016), which suggests that this symbiosis might be quite resilient to low levels of atmospheric N deposition.

Biotic regulators of cyanobacterial N_2-fixation activity

There is a high spatial and temporal variation of N_2-fixation activities supported by feathermosses (Gentili et al., 2005; Gundale et al., 2009; Jackson et al., 2011; Gundale et al., 2012a, 2012b; Jean et al., 2012; Sorensen et al., 2012). All these studies have focused on the role of extrinsic environmental factors in driving the observed spatial and temporal variability of N_2-fixation rates, but little is known about the role of the cyanobacterial community, as well as of other organisms inhabiting the moss, in contributing to this variability. Ininbergs et al. (2011) revealed that the diversity of *nifH* phylotypes was negatively correlated with N_2-fixation rates, suggesting that N_2-fixation might be performed by a few dominant cyanobacterial clusters. However, no attempts have been made to quantify the abundance or the N_2-fixation activity of individual cyanobacteria clusters occurring within the community. So far little attention has been paid to the composition and dynamic in the cyanobacterial community inhabiting *P. schreberi* and *H. splendens* and thus to how individual genera contribute to the total N_2-fixation rates measured.

Living and dead feathermosses as well as their associated organisms are called the 'bryosphere' (Lindo and Gonzalez, 2010). In addition to cyanobacteria, the microbial and invertebrate community of the bryosphere has a large diversity, and is organized in a complex food web (Lindo and Gonzalez, 2010). Apart from cyanobacteria, the mosses can host fungi, bacteria, tardigrades, nematodes, mites, and springtails (Jonsson et al., 2014; Kardol et al., 2016). For instance, more than 300 ascomycetes were found on the moss gametophyte (Dobbeler, 1997). Other fungi, arbuscular and ectomycorrhizal fungi as well as free-living zygomycetes and basidiomycetes can also be found in the bryosphere; these fungi can have a variety of interactions including pathogenic, parasitic, saprotrophic and commensal relationships with mosses (Carleton and Read, 1991; Davey and Currah, 2006; Kauserud et al., 2008). The fauna associated with the bryosphere includes a large diversity of amoebae, rotifers, ciliates, nematodes, rhizopods, moss mites and tardigrades (Jönsson, 2003; Anderson, 2006; Lindo and Winchester, 2006; Schatz and Behan-Pelletier, 2008). Many of these studies describe new species, suggesting that the bryosphere might be an understudied hotspot of microbial, microfauna- and mesofauna diversity. The trophic interactions in the bryosphere have been explored only in a single study (Kardol et al., 2016), where the authors found that microfauna feeds on cyanobacteria, and consequently re-

presses N_2-fixation activities. However, higher trophic levels (particularly microarthropods) feeding on the bacterivorous microfauna alleviate this top-down control. A better knowledge of the biotic factors, as well as investigations of the interactive effects of biotic and abiotic factors will contribute to a more complete understanding of the mechanisms underpinning N input and dynamics of boreal forests.

Effect of climate change on the feathermosses-cyanobacteria symbiosis

Considering that the boreal forest is the largest terrestrial biome on earth, accounting for 17% of the Earth's land surface (DeLuca and Boisvenue, 2012), and contains a large fraction of the terrestrial C pool (Hayes et al., 2011), it is crucial to investigate how climate change will affect this ecosystem. Climate change is predicted to result in temperature increases, elevated CO_2 concentrations, larger N deposition, and a longer growing season for the boreal ecosystems (Meehl et al., 2007). Specifically, the average temperature in the boreal forest has been predicted to increase by 2–8°C during this century (IPCC, 2007). This temperature elevation combined with raised CO_2 level has been proposed to increase the boreal forest's primary productivity (Cramer et al., 2001; Friedlingstein et al., 2006; Qian et al., 2010), thus sequestering more C in the ecosystem (De Vries et al., 2006; de Vries et al., 2014). However, even considering the effect of increased temperature on the productivity and C cycle, many studies have shown that productivity in boreal forest ecosystems may be constrained by the availability of N (Hungate et al., 2003; Reich et al., 2006; Jain et al., 2009; Gerber et al., 2010; Zaehle et al., 2010).

Despite the role of the feathermosses-cyanobacteria symbiosis in N cycling in the boreal ecosystem, little is known about how this process may respond to climate change (Bjerke et al., 2003; Deslippe et al., 2005; Sorensen and Michelsen, 2011). Global warming is predicted to cause drying events and rapid temperature variation, which have been shown to inhibit N_2-fixation by symbiotic cyanobacteria (Gundale et al., 2012b; Rousk et al., 2014a, Rousk et al., 2017). Nevertheless, the influence of other factors such as elevated atmospheric CO_2 needs more investigation (Lindo and Griffith, 2017). Altogether it is not known yet how the cyanobacteria and their hosts will react to the climate change, but this knowledge is crucial in predicting how biotic and abiotic factors will influence the rate of N_2-fixation and thus the total N and C input to the boreal forest. Given that CO_2 emissions are expected to double by the end of the current century combined with an increase in temperature (IPCC, 2007), determining the response of N_2-fixation to elevated CO_2 and its interactive effects with temperature is critical to deepen our understanding of the fate of boreal forests productivity. No studies have been performed to understand how the cyanobacterial community in symbiosis with feathermosses will be impacted by climate changes, and if those changes will affect the feathermosses as well as the N input in the boreal forests. Ultimately, such a study might yield insights in the climatic factors determining the rate and fate of fixed N to the boreal ecosystems in future times.

Aim of the thesis

The overall aim of this thesis was to expand our knowledge on the cyanobacteria in symbiosis with feathermosses, their evolution compared to other plant-cyanobionts, the molecular pathways involved in the interaction with the moss and the biotic regulators of their N_2-fixation activities. The approaches used in my PhD project integrate single gene studies, genomics, transcriptomics, and proteomics to arrive at evolutionary, genetic, physiologic and ecologic models of plant-cyanobacteria symbioses, with a specific focus on the feathermosses-cyanobacteria symbiosis. Specifically, my thesis can be divided into the following objectives:

1: Assess the genomic diversity of plant-symbiotic *Nostoc* strains and identify genomic changes associated with the evolutionary transition from free-living to different symbiotic lifestyles.

a. Survey the genomic and taxonomic diversity for multiple isolates of symbiotic *Nostoc* strains from the feathermosses *Pleurozium schreberi* and *Hylocomium splendens*, the liverwort *Blasia pusilla*, the water-fern *Azolla filicoides*, and the cycad *Macrozamia* sp.

b. Test the host-range of facultative symbiotic *Nostoc* strains, by symbiosis reconstitution experiments with multiple *Nostoc* isolates and the hosts, *B. pusilla* and *P. schreberi* (forming extracellular symbioses) and *Gunera manicata* (forming intracellular symbiosis).

c. Perform comparative genomics of the sequenced genomes of symbiotic *Nostoc* strains to investigate the evolutionary history of symbiotic competence present within the genus *Nostoc*. Explore the genomic retention and enrichment patterns of these lineages and conduct functional annotations to identify the cyanobacterial biological processes and/or pathways likely associated with the evolution of cyanobacteria-plant symbioses.

d. Infer the emergence and evolutionary history of plant-cyanobacteria symbioses by molecular dating using Bayesian relaxed molecular clock analysis on genome-wide phylogeny and provide novel insights into the origin of symbiotic *Nostoc* strains.

2: Understand the molecular nature of the symbiosis between cyanobacteria and feathermoss by comparative high throughput transcriptomic and proteomics approach.

a. Use laboratory-scale experiments in which *P. schreberi* and *Nostoc* strains are separated in two interaction phases allowing either chemical or also physical interactions between partners. Sample during the two interactions phases for transcriptomic/proteomic analyses to understand signaling required for symbiosis establishment and genes involved in establishing and maintaining a functional symbiosis.

b. Use an exoproteomic approach to study if the extracellular environment is changed during the chemical interaction between symbiotic *Nostoc* and the feathermoss *P. schreberi*.

c. Explore by transcriptomics during the physical contact phase between partners if the epiphytic location of *Nostoc* strains on the feathermosses involves different physiological and metabolic processes after colonization compared to the endophytic symbioses in the liverwort/hornwort and *Gunnera* sp. symbioses.

3: Explore the seasonal variation in cyanobacterial community composition and N_2-fixation activities of cyanobacterial communities associated with boreal feathermosses.

a. Gain insight on how and to what extent N_2-fixation rates performed by cyanobacteria in symbiosis with *P. schreberi* and *H. splendens* are related to the abundance and composition (as shown through *nifH* abundance) of the cyanobacterial community and whether this is consistent over the growing season. Specific *nifH* primers targeting individual cyanobacterial clusters commonly found in the community in symbiosis with *H. splendens* and *P. schreberi* were designed and used to quantify their abundance by quantitative PCR (qPCR).

b. Investigate how N_2-fixation activity (as shown through *nifH* expression quantified by qPCR) of individual cyanobacterial clusters within the community contributes to overall N_2-fixation rates and if variations of *nifH* expression predict the observed N_2-fixation rates.

c. Characterize the contribution of the different cyanobacterial *nifH* clusters to the N_2-fixation activity and identify the main cyanobacterial protagonists based on gene expression of the N input into boreal forest ecosystems.

4: Gain insights in the fate of the feathermoss-cyanobacteria symbiosis under the predicted climate change scenario by investigating the effect of elevated temperature and CO_2 on cyanobacterial community structure and composition, N_2-fixation activities and feathermoss growth.

a. Explore how this partnership cooperates under different temperature and CO_2 conditions, by following the changes in moss growth and cyanobacterial N_2-fixation rates. Further, examine changes of community structure and composition by targeted amplicon sequencing using the *nifH* gene, on reconstructed symbioses between *P. schreberi* and *Nostoc* strains isolated from feathermosses set at different level of cyanobacterial diversity.

b. Investigate the capacity of the isolated *Nostoc* strains to grow and fix N_2 under different temperature and CO_2 levels to evaluate if environmental filtering by these climatic factors selects specific cyanobacterial strains based on their ability to cope with these conditions.

c. Gain insights on how the relationship between cyanobacterial community structure and composition, N_2-fixation activity and the host growth might change in the future climatic conditions in boreal forests.

Comments on methodology

In **Paper I**, comparative genomics were performed on newly sequenced genomes of ten facultative symbiotic *Nostoc* strains, one obligate symbiotic *Nostoc* and one non-symbiotic *Nostoc* strain (Table 2). Genomic retention and enrichment patterns of these lineages was investigated to study the evolution of strategies from symbiotic and non-symbiotic cyanobacteria. Altogether, a set of 9,573 orthologous gene families covering eleven genomes of symbiotic *Nostoc,* the genome of the non-symbiotic strain *Nostoc sp.* CALU 996 (*N.* CALU 996) (Table 2) and two outgroup genomes was compiled based on an orthologous gene clustering analysis. Ancestral family sizes were inferred by assuming a probabilistic framework involving a phylogenetic birth-and-death model (Nei and Rooney, 2005) along a constructed rooted phylogeny for those strains. The model is described by lineage- and family-specific gene loss and duplication rates, coupled with a family gain process accounting for arrival by lateral gene transfer.

A major issue when studying changes in gene family size is the quality of the genome assemblies and annotations. Low sequence coverage in genome assemblies can lead to both erroneous addition and subtraction of genes. Genes can also be missing because of an incomplete coverage of the entire genome, with whole or parts of genes falling in unassembled portions of the genome (Hubisz et al., 2011). In addition, different sequencing technologies can create discrepancies in the genome assemblies and further ancestral family size inference (Denton et al., 2014). PacBio sequencing was used to obtain the genomes of five feathermoss symbiotic *Nostoc* isolates and of *N.* CALU 996 used in **Paper I and II,** while the four *Blasia* isolates used in **Paper I** were sequenced using MiSeq Illumina technology. In the PacBio technology, genomic DNA is sequenced in a single SMRT Cell which is expected to yield 350 Mb of long read (8-10 Kbp) sequences, longer reads than what MiSeq Illumina technology can provide (average fragment length 550 bp). A 30–50 times coverage of each feathermoss isolate's genome was obtained by PacBio sequencing and allowed for complete closure of the chromosome or a minimal number of scaffolds per genome as well as including any plasmids that might be present in the *Nostoc* strains (Table 2). Consequently, it was possible to close the chromosome of *N.* CALU 996. Gaps between the flanking regions of *N.* CALU 996 contigs were closed by PCR followed by sequencing of the single PCR product to confirm the junction. Thus, we can be confident that

the gene families identified as missing in *N*. CALU 996 but retained in the genomes of symbiotic competent strains were lost due to evolutionary processes and not because of incomplete sequencing of this strain. By contrast, genomes of the *Blasia* isolates are represented by a larger number of contigs which is making gap-filling PCR much more difficult (Table 2). Consequently, the gene flux analysis used to identify gene gain or loss in *Blasia* isolates can include errors due to the sequencing technology used. To reduce the bias, the analysis was focused on the 'lowest common ancestor' (LCA) of the intracellular *Nostoc* symbiont of *Gunnera* sp. Therefore, ultimately the gene flux analysis was restrained to genes lost/gained in all *Blasia* isolates as well as in the *Nostoc* sp. strain Moss2 (*N*. Moss2) and in *Nostoc punctiforme*. The genomes of the two last strains were assembled in few scaffolds (for *N*. Moss2) or closed (for *N. punctiforme*).

In **Paper II**, gene regulatory changes allowing cyanobacteria to form and maintain a symbiosis with feathermosses were determined using a proteogenomic approach comparing the feathermoss symbiotic strain *N*. Moss2, *N. punctiforme* and the non-symbiotic *N*. CALU 996. The gene expression patterns during the two contact phases were compared to the pattern of each strain when cultivated alone (i.e. in the absence of *P. schreberi*). Since the abundance of cyanobacterial mRNA is lower than that of rRNA from both partners and also lower than that of feathermoss mRNA, precautions were taken when sequencing and analysing the transcriptomic of cyanobacteria in physical contact with the host plant. The RNA libraries of cyanobacteria in physical contact with moss were treated as a mini-metatranscriptomes. Total RNA was depleted from bacterial and plant rRNA using the 50:50 (plant:microbial) Ribo-zero kit (Epicentre, Madison, WI, USA). Directional RNAseq libraries were generated from total RNA depleted for rRNA using the Epicenter ScriptSea2 kits (Epicentre). Three independent libraries (biological replicates) were generated for each cyanobacterial strain. All libraries were sequenced on an Illumina HiSeq2500 platform. A reference transcriptome for each species was generated using BBmap (https://sourceforge.net/projects/bbmap/) and compared to the genome of each cyanobacterial strains to put in the genomic context. HiSeq Illumina sequencing resulted in the detection of 84% of the total number of genes present in the genome of *N. punctiforme* and 55% for the genome of *N*. Moss2. The coverage obtained by the transcriptome in the physical contact phase was acceptable for *N. punctiforme*, but the coverage of *N*. Moss2 can be considered as problematic. Consequently, cyanobacterial transcripts representing genes that are putatively important for the symbiosis, but expressed at low levels, could have remained undetected. It has to be considered that in **Paper II,** only the set of 4,071 candidate genes shared by *N. punctifome* and *N*. Moss2 was of interest, and 80% of this set was detected in the transcriptomes of both cyanobacteria in physical contact with *P. schreberi*.

Taxon	Primary host-plant	Sequencing technology	Size (Mb)*	GC %*	CDS*	rRNA*	tRNA*	ANI**	Nb of contigs
Nostoc punctiforme ATCC 29133 (1)	Cycad, *Macrozamia* sp.	Sanger	9.05	41	6690	12	88	-	Genome closed
Nostoc sp. Moss2	Feathermoss, *P. schreberi*	PacBio	9.16	42	7876	9	67	88.79	24 contigs
Nostoc sp. Moss3	Feathermoss, *P. schreberi*	PacBio	9.10	41	7471	9	96	83.05	12 contigs
Nostoc sp. Moss4	Feathermoss, *H. splendens*	PacBio	8.97	41	7610	12	69	84.70	26 contigs
Nostoc sp. Moss5	Feathermoss, *P. schreberi*	PacBio	7.24	41	6241	12	87	79.52	67 contigs
Nostoc sp. Moss6	Feathermoss, *H. splendens*	PacBio	7.14	41	6119	12	87	79.56	11 contigs
Nostoc sp. KVJ2	Liverwort, *Blasia pusilla*	Illumina MiSeq	8.69	41	8724	5	97	89.02	417 contigs
Nostoc sp. KVJ10	Liverwort, *Blasia pusilla*	Illumina MiSeq	10.39	41	14132	2	101	88.61	4501 contigs
Nostoc sp. KVJ20	Liverwort, *Blasia pusilla*	Illumina MiSeq	9.18	41	9267	4	89	88.76	549 contigs
Nostoc sp. KVS11	Liverwort, *Blasia pusilla*	Illumina MiSeq	8.30	41	8993	8	81	89.04	1269 contigs
Nostoc azollae 0708 (2)	Water-fern, *Azolla filiculoides*	Sanger	5.48	38	5321	12	44	78.44	Genome closed
Nostoc sp. CALU 996 (3)	Free-living	PacBio	7.98	41	7309	13	60	-	Genome closed

Table 2. Genomic features of the symbiotic Nostocales. * values are taken from the integrative microbial genomes (IMG) data management and analysis system (Markowitz et al., 2014) for all species but *N.* KVJ2, KVJ10, KVJ20 and KVS11 where values are coming from PATRIC (Wattam et al., 2017). **Pairwise ANI is in comparison with *N. punctiforme*. (1) Meeks et al. (2001); (2) Ran et al. (2010), (3) Warshan et al. (2017).

An alternative to the approach chosen in **Paper II** could have been to generate RNA libraries depleted from eukaryotic RNA by using polyadenylated magnetic beads; another option would have been to go for a larger number of reads. In any case, the transcriptome of the feathermoss in physical contact with cyanobacteria was also generated from the same libraries and will be used for further studies.

In **Papers III and IV**, *nifH* relative abundance and/or expression of individual cyanobacterial clusters within the community was explored. The *nifH* gene was used as molecular marker due to its key role in the N_2-fixation process through coding for a structural component (Fe-protein) of the nitrogenase enzyme, and because a large database of sequences is available due to its widespread use as a molecular marker for diazotrophs. For **Papers III and IV**, some caveats have to be taken into consideration when using *nifH* as a

molecular marker. The use of the *nifH* gene as molecular marker in **Papers III and IV** enabled a higher taxonomic resolution of the genetic variation of *nifH* phylotypes composing the cyanobacterial communities than any other marker studied to date for this system (Ininbergs et al., 2011). Han et al. (2009) evaluated the use of other molecular markers that could have been used to study cyanobacterial diversity, such as *hetR*, *rpoC1*, *rbcLX* or 16S rRNA, and concluded that the gene trees of these molecular markers are not congruent. In studies using the 16S rRNA gene, it was shown that this marker is not able to distinguish between closely related *Nostoc* strains (Papaefthimiou et al., 2008a; Cutler et al., 2017). In the study by Cutler et al. (2017), where the 16S rRNA gene was used as target for amplicon sequencing of the microbial community associated with *P. schreberi* samples, the authors could only distinguish cyanobacterial sequences at the genus level, which was not the taxonomic level required to answer the aims of **Papers III and IV**. Nevertheless, the $tRNA^{Leu}$(UAA) intron (*trnL*) could have been used as marker gene, since it is commonly used to study symbiotic *Nostoc* strains in lichen and bryophytes (for an exhaustive review see Rikkinen, 2013). However, a drawback concerning the use of *trnL* is that there are no studies that used this marker to investigate the cyanobacterial community associated with feathermosses, thus no database was available to retrieve cyanobacterial phylotypes which was a requirement to answer the aims of **Papers III and IV**. Another caveat is that the use of specific primers for cyanobacterial *nifH* excludes the putative quantification of the presence of other diazotrophs. One of the aims of **Paper III** was to explore how *nifH* expression by the cyanobacterial community can explain the total N_2-fixation activity. Thus, the question of the *nifH* expression of other N_2-fixers in the microbial community, and whether their expression of this gene could explain variations in N_2-fixation activity, was not assessed. The choice of *nifH* gene as a proxy of N_2-fixation fixation activity restrict the interpretation of the results to the iron-molydnemum type of nitrogenase. Thus, if some cyanobacteria are shifting to the iron-vanadium alternative nitrogenase due to molybdenum limitation, this was not accounted in **Paper III**. Investigating if the *vnf* genes, encoding for the vanadium-iron nitrogenase, has the same taxonomic resolution that the *nif* genes, is a first step to be able to perform similar investigations as performed in **Paper III**.

Moreover, a third caveat is that since cyanobacteria can have more than one copy of *nifH* gene per genome and polyploidy i.e. the presence of multiple genome copies per cell (Sargent et al., 2016). The use of *nifH* copy numbers as a measure of abundance of cyanobacterial cluster in **Paper III and IV** can result in overestimates. In these Papers, a 1:1 cell:genome ratio and 1:1 *nifH*:genome ratio were assumed, in reality these ratios can differ overtime, when talking about the polyploidy, and between cyanobacteria strains for both ratios. Thus, the results of the *nifH* abundance in **Paper III and IV** should be carefully interpreted, and in the future assessing potential biased coming from

polyploidy in cyanobacterial genome should be investigated by for instance DAPI staining of the cyanobacterial DNA. Moreover, in **Paper IV** the nucleotide sequences of six *nifH* phylotypes were identical to the *nifH* gene of several inoculated *Nostoc* strains, meaning that one phylotype represented more than a single strain. This suggests that even though the *nifH* gene has high taxonomic resolution among molecular markers used to study feathermoss-associated cyanobacteria (Ininbergs et al., 2011), the actual number of cyanobacterial strains associated with the moss cannot be estimated solely by using the *nifH* gene. Nevertheless, *nifH* phylotyping performed in **Paper IV** was shown to act as a proxy of number of strains present in a cyanobacterial community and remains a valid approach to study cyanobacterial diversity associated with feathermosses.

Results and Discussion

This thesis intends to gain insight on cyanobacteria in symbioses with plants, with a specific focus on the ecologically important feathermoss-cyanobacteria symbiosis. The four primary objectives of the thesis were:

1) Create genetic and evolutionary understandings of plant-symbiotic cyanobacteria encompassing three aspects of their diversity; the symbiotic cyanobacteria genomic diversity (**Paper I** and **II**), their taxonomic diversity (**Paper I, II, III** and **IV**), and their diversity in symbiotic performance (**Paper III** and **IV**).

2) Generate insights on the interaction between plant and cyanobacteria, through a better comprehension of the molecular pathways involved in the establishment and maintenance of cyanobacterial-feathermosses symbiosis (**Paper II**).

3) Broaden the comprehension on the relationship that cyanobacteria have with their host plant, by *in situ* (**Paper III**) and laboratory-scale (**Paper IV**) experiments using feathermosses and cyanobacteria partners.

4) Characterize the temporal variation of the cyanobacterial community composition associated with feathermosses (**Paper III**) and if this composition will change in the future boreal forests climatic conditions, with specific focus on the effect of temperature and CO_2 level (**Paper IV**).

Evolutionary transition from free-living *Nostoc* to different symbiotic lifestyles with plants

Reconstitution experiments with eleven *Nostoc* isolates and the liverwort *Blasia pusilla*, the feathermosses *Pleurozium schreberi* (forming extracellular symbioses) and the angiosperm *Gunnera manicata* (forming intracellular symbiosis) were performed in order to investigate their ability to colonized different host plants (**Paper I**). Cyanobacteria isolated from the bryophytes, *P. schreberi* and *B. pusilla*, were capable of switching host and establishing an extracellular symbiosis with both plants. However, the capacity to form an intracellular symbiosis with *G. manicata* was only observed for the model symbiotic strain *Nostoc punctiforme* ATCC 29133 (also referred as PCC 73102), isolated from the cycad *Macrozamia* sp., the isolates from *B. pusilla* and a single feathermoss isolate, *Nostoc* sp. strain Moss2 (*N.* Moss2) (Figure

6). Differences in symbiotic behavior between those two types of feathermoss symbionts were observed in **Paper IV**. Higher abundance of *N.* Moss2 explained a higher overall measured N_2-fixation and increased feathermoss growth compared to other *Nostoc* strains restricted to extracellular symbioses. The phylogenetic analysis of concatenated protein alignments was consistent with the symbiotic reconstruction experiment and grouped all *Nostoc* strains able to enter an intracellular symbiosis with *G. manicata* as well as extracellular symbiosis into a monophyletic clade referred as Intra/Extra clade (Figure 6). The other clades of symbiotic *Nostoc* strains that are only able to enter extracellular symbioses formed two polyphyletic clades.

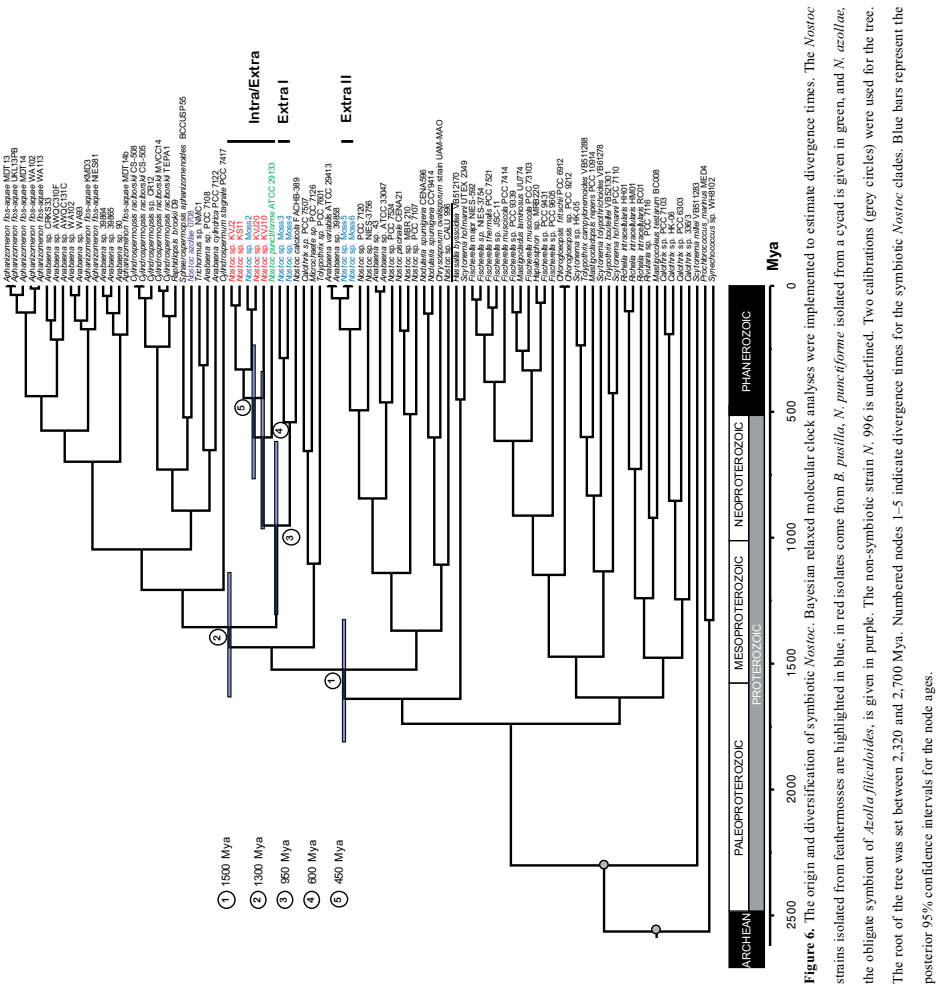


Figure 6. The origin and diversification of symbiotic *Nostoc*. Bayesian relaxed molecular clock analyses were implemented to estimate divergence times. The *Nostoc* strains isolated from feathermosses are highlighted in blue, in red isolates come from *B. pusilla*, *N. punctiforme* isolated from a cycad is given in green, and *N. azollae*, the obligate symbiont of *Azolla filiculoides*, is given in purple. The non-symbiotic strain *N.* 996 is underlined. Two calibrations (grey circles) were used for the tree. The root of the tree was set between 2,320 and 2,700 Mya. Numbered nodes 1-5 indicate divergence times for the symbiotic *Nostoc* clades. Blue bars represent the posterior 95% confidence intervals for the node ages.

The two "Extracellular clades" (referred as Extra clades) were composed of feathermoss isolate *Nostoc* strains: *Nostoc* sp. Moss3 and *Nostoc* sp. Moss4

and strains: *Nostoc* sp. Moss5 and *Nostoc* sp. Moss6 (hereafter abbreviated *N.* Moss3, 4, 5 and 6, respectively).

By implementing phylogenomic and Bayesian relaxed molecular clock analyses, the phylogeny and age divergences of symbiotic *Nostoc* was estimated (Figure 6 and 7). The first divergence was estimated to have taken place ca. 1,500 Mya, when the cyanobacterial feathermoss isolates *N.* Moss5 and *N.* Moss6 (Extra II clade), as well as the non-symbiotic strain *Nostoc* sp. CALU 996 (*N*. CALU 996) diverged from the other clades of symbiotic *Nostoc* strains. The second divergence between symbiotic *Nostoc* strains was estimated at ca. 1,300 Mya, when *Nostoc azollae*, the obligate symbiont of *Azolla filiculoides*, diverged from the Intra/Extra clade and the Extra I clade. Finally, the last divergence between the Intra/Extra clade and the Extra I clade was dated at ca. 950 Mya. In addition, the origin of the Intra/Extra clade, was estimated at ca. 600 Mya, as well as within this clade, the more recent divergence between the cycad isolate *N. punctiforme* and the bryophyte *Nostoc* isolates at ca. 450 Mya (Figure 6 and 7). Surprisingly, the emergence of the Intra/Extra clade predated the origin of land plants in the Mid-Ordovician period at ca. 471 Mya, as well as the emergence of the host genus *Gunnera* dated to ca. 115 Mya (Figure 7) (Vekemans et al., 2012; Magallón et al., 2013; Edwards et al., 2014). The earliest direct fossil evidence of an intracellular cyanobacterial symbiosis is the observation of cyanobacterial filaments in prostrate axes of *Aglaophyton major*, an early vascular land plant, which has been dated at ca. 400 Mya (Krings et al., 2009). Due to the lack of observation of heterocyst or akinete structure in these fossils it was hypothesized that Oscillatoriales and not Nostocales were the cyanobacteria associated with *A*. *major* (Krings et al., 2009). These results suggest that the intracellular lifestyle of *Nostoc* appeared with another host than a member of the Gunneridae. Another intracellular symbiosis involving *N. punctiforme* is the one with *Geosiphon pyriformis,* a glomeromycotan fungus (Schüßler et al., 1994). These results could be interpreted to mean that the lowest common ancestor (LCA) of the Intra/Extra clade was probably capable of forming intracellular symbioses with early-land plants and/or fungi, and that the capacity was lost during evolution with the exception of *Geosiphon* and *Gunnera* sp.

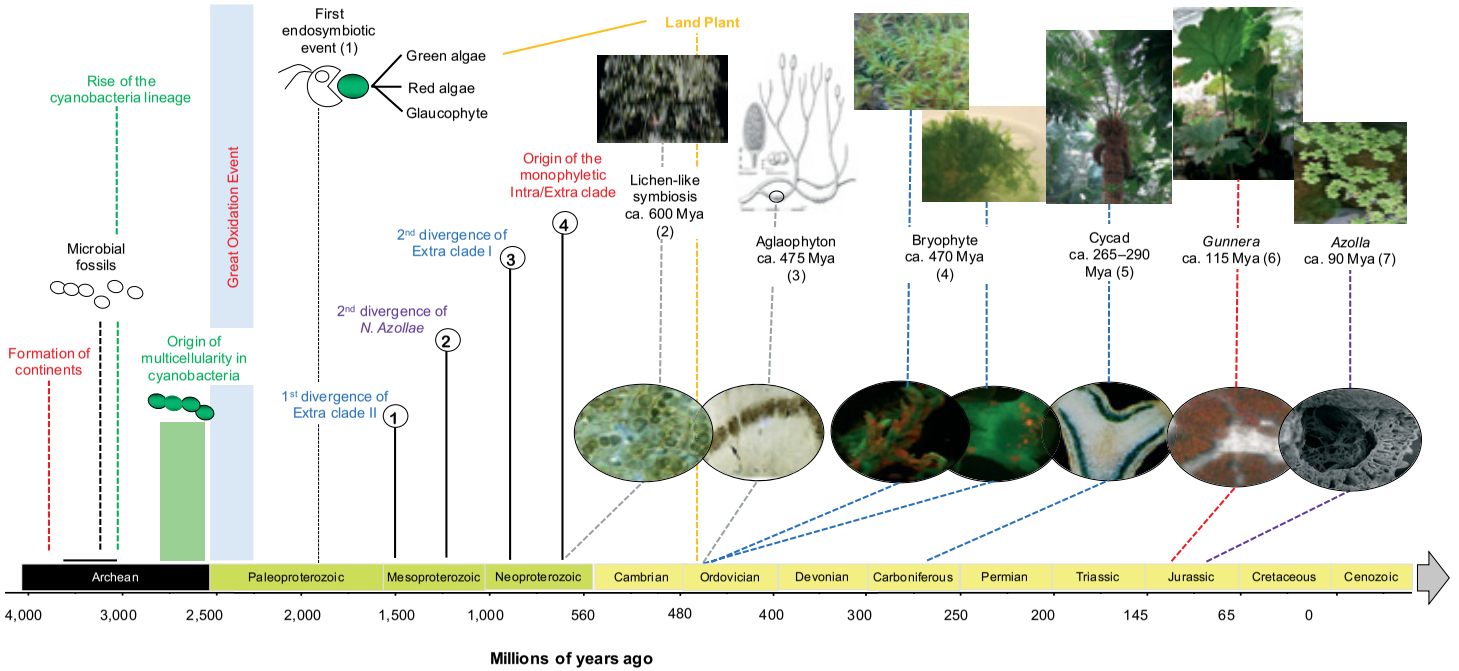


Figure 7. A possible scenario of the evolution of *Nostoc* in symbiosis with plants. Image shows the different extant terrestrial symbioses involving *Nostoc* sp., and the cyanobacteria symbiont of the early land plant *Aglaophyton major* from the Lower Devonian Rhynie Chert (Krings et al., 2009). Photos illustrating the lichen-like symbiosis represent the bipartite cyanolichen *Leptogium* sp. and its *Nostoc* symbiont (Rikkinen, 2017). Divergence time and origin of symbiotic *Nostoc* were estimated from bayesian relaxed molecular clock analyses (see Figure 6). (1) Sánchez-Baracaldo et al. (2017) (2) Yuan et al. (2005) (3) Krings et al. (2009) (4) Edwards et al. (2014); Brown et al. (2015) (5) Brenner et al. (2003); Condamine et al. (2015) (6) Vekemans et al. (2012) (7) Metzgar et al. (2007). Photos of *Aglaophyton major* from (Krings et al., 2009), and cyanolichen from Rikkinen (2017) are adapted with permission. Additional photos from Ulla Rasmussen and Denis Warshan.

Molecular pathways required in the establishment and maintenance of a symbiosis between *Nostoc* and plants

A set of transporters is shared in symbiotic Nostoc *strains*

A gene flux analysis was performed to identify gene families that were gained/expanded or lost/contracted along a species tree composed of symbiotic and non-symbiotic *Nostoc* strains (**Paper I)**. This analysis allowed to define a set of gene families that might be involved in the establishment and maintenance of cyanobacterial-plant symbioses. The non-symbiotic strain *N.* CALU 996 lost/contracted 268/35 gene families and among those, 170 (55%) were retained in the genomes of symbiotic strains. The relatively small set of genes not retained in *N.* CALU 996 suggest that only a few molecular pathways are involved in the establishment and maintenance of cyanobacterial-plant symbioses. The LCA, lowest common ancestor, of the Intra/Extra *Nostoc* strains gained/expanded 171/41 gene families, which also represent a small part of the total number of gene families in the LCA's genome, containing 4,997 gene families.

Global transcriptional and protein profiles were explored for the isolate *N.* Moss2, *N. punctiforme*, and *N.* CALU 996 under three conditions: in isolation, in chemical contact (early colonization phase), and in physical contact (late colonization phase) with the feathermoss *P. schreberi* (Figure 9, **Paper II**). Some of the families identified as specifically retained among symbiotic *Nostoc* strains over the course of evolution, were found as differentially regulated at the transcriptional and/or protein level when in contact with the host *P. schreberi* (Figure 8 and 9; **Papers I** and **II**). For instance, the genomes of facultative symbiotic *Nostoc* strains (Figure 6) were enriched in gene families encoding substrate binding periplasmic proteins associated with the transport of aliphatic and alkane sulfonates, sulfate, phosphate, branched-chain amino acids and a gene encoding an ammonium importer (Figure 8). Transcriptional up-regulation of gene families encoding phosphate and sulfate transporters was observed for *N.* Moss2 and *N. punctiforme* in symbiosis with the feathermoss *P. schreberi* (Figure 9; **Paper II**). The genomic acquisition of these additional transporters suggests an adaptation to a nutrient-rich environment offered by the host. Deficiency in micro- and macronutrients are known to limit N_2-fixation activity in terrestrial ecosystems (Vitousek et al., 2013) and in at least one study, phosphorus was shown to be a limiting factor for N_2-fixation activities of feathermoss associated cyanobacteria (Rousk et al., 2017). These nutrients should be available in sufficient quantities in the host environment to sustain cyanobacterial N_2-fixation. A branched-chain amino acid transporter was also found to be conserved among all symbiotic *Nostoc* strains (Figure 8; **Paper I**). While so far, no results have been reported on the role of branched-chain amino acid transporters in cyanobacterial-plant symbioses, it

is well known that in legume root nodules, the plant supplies the symbiotic rhizobia (bacteroids) with branched-chain amino acids (BCAAs) while rhizobial BCAA biosynthesis is downregulated (Prell et al., 2010). Interestingly, the comparison of the free-living and symbiotic transcriptomes of *N. punctiforme* and *N.* Moss2 showed that BCAA biosynthetic pathways are also down-regulated in cyanobacterial symbioses (**Papers I** and **II**). Hence, this feature seems to be shared between two independently evolved symbioses between plants and diazotrophs, the feathermoss-cyanobacterial and rhizobial symbioses. This might be due to the fact that BCAA biosynthesis can work as an electron sink (Shimizu et al., 2010) while N_2-fixation requires a high reductive potential. All facultative symbiotic *Nostoc* strains were enriched in transporters of alkane sulfonate and other aliphatic sulfonates but also retained specific catabolic pathways for aliphatic sulfonates such as taurine, and alkane sulfonates, to generate sulfite (Figure 8; **Papers I** and **II**). Alkane sulfonate monooxygenases and a taurine catabolism dioxygenase were specifically retained in the genome of all facultative symbiotic *Nostoc* strains, allowing the anabolic incorporation of sulfonate-derived sulfite into L-cysteine (Figure 8; **Paper I**). The importance of these functions for the symbiosis is underlined by the fact that the gene families of aliphatic sulfonate transporters and monooxygenases was found to be highly transcriptionally upregulated during physical contact with feathermoss in *N. punctiforme* and *N*. Moss2 (Figure 9; **Paper II**). This may indicate a parallel with the symbiosis between a marine *Roseobacter* clade bacterium and the diatom *Thalassiosira pseudonana* where an aliphatic sulfonate is supplied by the diatom to the bacterium (Durham et al., 2015). So, the feathermosses might transfer aliphatic sulfonates to their cyanobiont in exchange for fixed N, a transfer system which might be shared by other eukaryote-prokaryote symbioses. Interestingly, the LCA of the Intra/Extra clade specifically gained genes encoding proteins involved in the assimilation of sulfite, a homoserine-*O*-acetyltransferase and an *O*-acetyl-L-homoserine sulfhydrylase to synthetize L-methionine from hydrogen sulphite and *O*-acetyl-L-homoserine (Figure 8). This is yet another parallel with legume/rhizobia symbioses in that bacterial methionine biosynthesis has been shown to be essential for *Sinorhizobium meliloti* to survive in root nodules (Campbell et al., 2006; Taga et al., 2007; Taga and Walker, 2010).

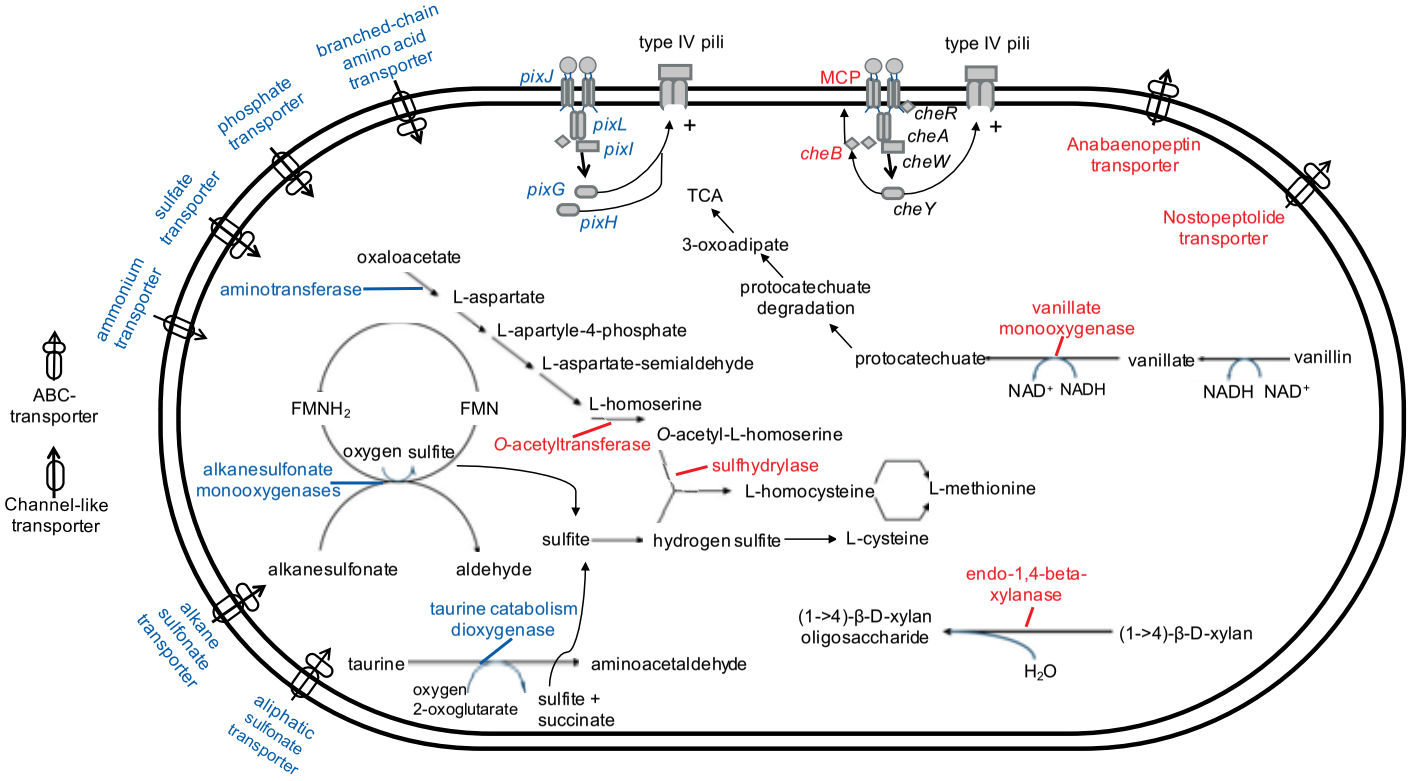


Figure 8. Overview of the enriched pathway in all facultative symbiotic *Nostoc* (blue) and specifically gained by the LCA of intracellular competent *Nostoc* strains (red). FMN, flavin mononucleotide; MCP, modular cyanobacteriochrome–methyl-accepting chemotaxis protein; TCA, tricarboxylic acid cycle.

Symbiotic Nostoc *strains are enriched in chemotaxis and motility pathways*

Cyanobacteria require the differentiation of vegetative filaments into hormogonia in order to colonize a new host (Rai et al., 2000). There is evidence suggesting that cyanobacterial interactions with their host plants are mediated by chemotaxis-related signal transduction systems (Duggan et al., 2013; Campbell et al., 2015). The gene flux analysis highlighted that all facultative symbiotic *Nostoc* strains retained the *pix* locus, which contains chemotaxis and motility genes (Figure 8; **Paper I**). Further, the importance of the *pix* locus and six other loci containing chemotaxis and motility-related genes were shown to be up-regulated during the establishment and/or maintenance of the feathermoss symbiosis (Figure 9; **Paper II**). In addition, the *pix* genes were induced during both the early colonization phase when chemical signals are exchanged between the partners, and during the later phase when the cyanobacteria are physically associated with *P. schreberi* (Figure 9; **Paper II**). The LCA of the Intra/Extra clade specifically gained one gene family encoding a modular cyanobacteriochrome-methyl-accepting chemotaxis protein (MCP) and expanded another MCP gene family and a *cheB*–like gene family which catalyzes the demethylation of specific methylglutamate residues introduced into chemoreceptors by the chemotaxis protein methyltransferase CheR (Figure 8; **Paper I**). Deletion of those two genes as well as other linked *cheAWR* and MCP genes from the genome of *N. punctiforme* showed that this locus is not involved in either hormogonium differentiation, motility, or in phototaxis (Campbell et al., 2015). This result together with our findings suggests that this locus might be specifically involved in the establishment or maintenance of a symbiosis. The analysis of global transcript abundance and protein expression showed that those Intra/Extra clade-enriched chemotaxis genes were not specifically up-regulated during the symbiosis with the feathermoss *P. schreberi* (**Paper II**), suggesting that this molecular pathway might be specifically regulated during the *Gunnera*-cyanobacteria symbiosis.

The lowest common ancestor of the Intra/Extra clade gained/enriched pathways for the degradation of vanillin and hemicellulose

Among the gene families specifically enriched by the LCA of the Intra/Extra clade were genes involved in the degradation of vanillin (**Paper I**), which is a plant phenolic compound commonly found in plant exudates (Li et al., 2010). All members of the Intra/Extra clade contain genes encoding enzymes in the pathway for the degradation of vanillin and vanillate (Figure 8; **Paper I**). The vanillate monooxygenase which generates protocatechuate from vanillate was gained by the LCA. In addition, the complete pathway for the degradation of protocatechuate to 3-oxoadipate is present in all symbiotic strains, where 3-oxoadipate is further broken down to acetyl-CoA, which enters the

TCA cycle (Figure 8; **Paper I**). Vanillate can be an important source of energy and carbon for microorganisms (Nardi et al., 2003; MacLean et al., 2006; Varman et al., 2016); e.g., the symbiotic root nodule bacterium *Bradyrhizobium japonicum* is able to aerobically catabolize vanillate and protocatechuate (Ito et al., 2006). One degradation pathway for hemicellulose was specifically gained in the LCA of the Intra/Extra clade in that an endo-1,4-beta-xylanase involved in xylan degradation was acquired (Figure 8). Altogether to be able to break down parts of the plant cell wall – hemicelluloses – such as xylan, may be a key feature in the intracellular colonization of *Gunnera* sp. by *Nostoc*. In addition, xylans can represent a substantial source of nutrition for bacteria able to degrade this substrate (Kulkarni et al., 1999; Saha, 2003; Dodd and Cann, 2009). Additionally, the vanillate monooxygenase and the glycoside hydrolase for hemicellulose degradation were highly upregulated in *N.* Moss2 during physical contact with *P. schreberi*, suggesting vanillate and xylan as C sources for the Intra/Extra strains when in symbiosis with different host plants (**Paper II**). Thus, being able to use xylans and vanillate as C sources can give an advantage for strains belonging to the Intra/Extra clade over strains in the Extra clades, in their ability to occupy a different niche in the cyanobiont community.

Transcriptional and post-transcriptional regulation in Nostoc *strains during chemical or physical contact with feathermosses*

Two gene families involved in nitric oxide (NO) signalling are also retained in the genome of facultative symbiotic *Nostoc* strains (**Paper I** and **II**). Genes encoding a heme-nitric oxide/oxygen binding (H-NOX) protein that selectively binds NO, and a NO-associated protein annotated as a histidine kinase were up-regulated during chemical and physical contact. This suggests a role of NO in signalling during establishment and maintenance of plant-cyanobacteria symbioses (Figure 9; **Paper II**). Rao et al. (2015) showed that *Silicibacter* sp. TrichCH4B and the marine cyanobacterium *Trichodesmium erythraeum* have an NO-mediated mechanism for symbiosis, in which *T. erythraeum* produces a chemical signal, inducing NO production in the symbiont. NO activates the NO synthesis pathway of *Silicibacter* sp. TrichCH4B, leading to higher levels of cellular c-di-GMP controlling adhesion and biofilm formation. Increased NO concentrations might lead to up-regulation of the H-NOX gene and to c-di-GMP synthesis genes in the symbiotic *Nostoc* strains. Biofilm formation is often important to host-symbiont communication especially when it is mediated by diffusible signalling molecules (Norsworthy and Visick, 2013), so biofilm formation might favour host-symbiont NO-mediated communication and enhance nutrient exchange. If the source of NO is the host, it might allow NO-mediated signalling from the plant to guide cyanobacteria migration and colonization of new plant tissue. Consistent with

this hypothesis it was shown that high levels of NO are produced in protonema cells during the growth of moss (Medina-Andrés et al., 2015).

The transcriptomic data of *Nostoc* strains (*N.* Moss2 and *N. punctiforme*) in physical contact with *P. schreberi* highlighted that cyanobacteria in symbiosis with feathermosses have a distinct metabolic regulation relative to endophytic cyanobacteria-plant symbioses (Figure 9; **Paper II**). In endophytic symbioses, cyanobacterial heterocyst frequency and the N_2-fixation activity increases when in contact with the host (Adams and Duggan, 2012). No statistically significant up-regulation of *nifH* or *hetR* genes was found in cyanobacteria when associated with *P. schreberi* relative to when in free-living stage, indicating that the cyanobacteria do not increase their N_2-fixation activity when growing on the moss. The expression of *nifH* in physical contact also remains constant compared to when grown without the moss. Despite lack of differential expression when comparing free-living and physical contact treatments, both *hetR* and *nifH* genes were among the 10% most abundant transcripts after colonization of the moss. In other cyanobacterial symbioses, except the cycad symbiosis, ammonium is released by *Nostoc* strains, achieved partly by a modulation of glutamine synthetase (GS) activity, with a reduction from 70% to 15% of *in vitro* activity of the enzyme during symbiosis with *Gunnera* and *Anthoceros*, respectively (Meeks, 2009). *glnA* (GS) and glutamate synthase (GOGAT) *gltB* transcripts are highly abundant post-colonization and statistically indistinguishable from the free-living condition (**Paper II**). The results could indicate that the cyanobacteria may not transfer ammonium to the moss, but rather amino acids, as in the cycad-cyanobacteria symbiosis (Costa and Lindblad, 2002).

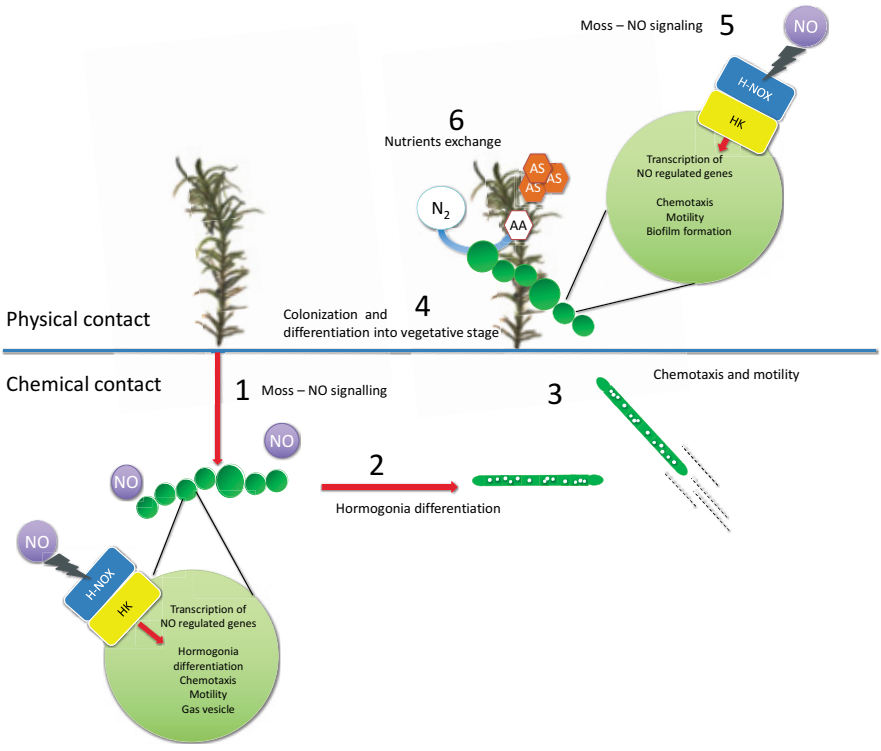


Figure 9. Conceptual model for establishment and maintenance of a cyanobacteria-feather-moss symbiosis. Putative mechanisms involved during chemical contact between *P. schreberi* and symbiotic cyanobacteria and molecular steps leading to the establishment of the symbiosis, physical contact. (1) Moss releases NO into the environment, which is sensed by the H-NOX fused with a histidine kinase (HK). (2) NO signaling pathway triggers hormogonia differentiation (*hrm* locus) and gas vesicle formation. (3) Chemotaxis and motility (*ptx*, *pix* and *hmp* loci and *pil* and *hsp* loci, respectively). (4) Cyanobacteria colonize the moss and differentiate into their vegetative stage. (5) The host signals to the cyanobacteria using NO for spatial localization and re-direction of the hormogonia. Increased NO signaling induces biofilm formation to favour host-symbiont NO-mediated communication and enhance nutrient exchange. (6) Production of aliphatic sulfonate (AS) compounds and transferred of these from the host to the cyanobacteria. The cyanobacteria might provide N as amino acids (AA) to the moss. Adapted from Warshan et al. (2017).

Relationship between cyanobacterial community composition and N_2-fixation activity, insights from field study experiments

Diazotrophs associated with pleurocarpous feather mosses play a key role in the N cycle of boreal forests (DeLuca et al., 2002; Zackrisson et al., 2004; Turetsky et al., 2012; Lindo et al., 2013; Jonsson et al., 2014). The majority of studies on this topic report N_2-fixation rate variations between the two

growing seasons in June and September (Deluca et al., 2002) and forest stands (Zackrisson et al., 2004; Stuiver et al., 2015), and most of these observed differences have been attributed to local environmental conditions (Smith, 1984; Zackrisson et al., 2004; Gentili et al., 2005; Gundale et al., 2009; Jackson et al., 2011; Gundale et al., 2012a, 2012b; Jean et al., 2012; Sorensen et al., 2012; Rousk et al., 2013). N_2-fixation rates in mosses collected across nine forest sites located at the Reivo forests in the northern Swedish boreal zone, near Arvidsjaur, in June and September were measured. Temporal and host-dependent variations were found (**Paper III**). The N_2-fixation rates across forest sites were higher in September than June for both *H. splendens* and *P. schreberi* (**Paper III**). To explore the diurnal variation in N_2-fixation activity, the site showing the highest variation between the two seasons was selected. The two moss species were collected at the time of the day when the N_2-fixation rates peaked and analyses of the cyanobacterial community composition, through *nifH* abundance, and of N_2-fixation activity through *nifH* gene expression were performed by qPCR. The results showed temporal and host-dependent variations of the cyanobacterial community composition, *nifH* gene abundance and expression (Figure 10, **Paper III**). The total relative cyanobacterial *nifH* gene abundance was higher in September than in June for *H. splendens* as well as higher on *H. splendens* compared to that on *P. schreberi* (Figure 10, **Paper III**). Therefore, these results not only provide evidence that composition is host specific as previously suggested by Ininbergs et al. (2011), but also suggest a promiscuous symbiosis with a mixed community changing over time. Variation in N_2-fixation rates was explained by higher *nifH* gene expression level by individual clusters rather than higher *nifH* gene abundance or differences in cyanobacterial community composition. With the exception of the cyanobacterial community found in September on *H. splendens*, the *Stigonema* cluster made up less than 29% of the total cyanobacterial community, but accounted for the majority of *nifH* gene expression (82%–94% of total *nifH* expression), irrespective of sampling date or moss species (Figure 10). Stepwise multiple regressions showed that the temporal variations in N_2-fixation rates could to a large extent be explained by variations in *nifH* expression of the *Stigonema* cluster (**Paper III**). The dominance of the *Stigonema* cluster on *H. splendens* in September has also been observed in a previous study from the boreal forest of Quebec, Canada where *Stigonema* sp. was the most abundant genus on *H. splendens* compared to *P. schreberi* during the late autumn season, i.e. September - October (Houle et al., 2006). Additionally, a higher number of phylotypes affiliated to the *Stigonema* cluster was also observed on *H. splendens* compared to *P. schreberi* in the work of Ininbergs et al. (2011). Positive correlations of N_2-fixation rates were identified with the *nifH* gene abundance of the *Stigonema* cluster and the *nifH*2 cluster, and the *nifH* gene expression of the *Nostoc* clus-

ter I notwithstanding its poor contribution in explaining N_2-fixation rate variations. The dominance of *Nostoc* species (represented in the *Nostoc* cluster I and the *nifH2* cluster) in the cyanobacterial community is therefore evident. However, their contribution to the *nifH* gene expression pool is minor. As a result, *Nostoc* species could most likely be considered as a smaller contributor with regard to N input into boreal forests compared to *Stigonema*. Thus, *Stigonema* species are potentially the most influential N_2-fixers in symbiosis with boreal forest feathermosses.

Nevertheless, results from **Paper IV** indicate that different *nifH* phylotypes can have different symbiotic performance i.e. be differentially correlated with N_2-fixation activity and moss growth rate (MGR). For instance, the abundance of *nifH* phylotypes related to the Intra/Extra clade cyanobacterial strain *N.* Moss2 were shown to be positively correlated with increased N_2-fixation activity and growth rates of *P. schreberi* (**Paper IV**). In addition, the strains *N*. Moss5/6 belonging to the Extra clade II were found to exhibit the highest N_2-fixation rate when in isolation under elevated temperature and CO_2 levels and during symbiotic growth under the same conditions the *nifH* abundance associated with this cluster was positively correlated with N_2-fixation rates. However, in contrast with *N.* Moss2, relative abundance of *N*. Moss5/6 phylotypes was not correlated with higher MGR, which suggest that *N*. Moss5/6 strains do not contribute as much as *N*. Moss2 to the MGR by transferring N products (**Paper IV**). In the field study, the *Stigonema* cluster was found to be potentially the main N_2-fixer in the cyanobacterial community (**Paper III**), but in the lab-experiment conducted in **Paper IV**, some *nifH* phylotypes belonging to this cluster were shown to be differentially correlated with N_2-fixation and MGR. These results suggest that symbiotic performances i.e. contribution to N_2-fixation activity and MGR, differ also at the phylotype level and not only at the cluster level. Besides, when these results are interpreted in the context of the phylogenetic inference performed in this thesis (Figure 6, **Paper I**), they suggest that feathermoss cyanobionts belonging to the Extra clades such as *N*. Moss5/6 perform better in terms of N_2-fixation than the members of the Intra/Extra clade, but the cyanobacteria contributing to MGR were mainly members of the Intra/Extra clade and the *Stigonema* cluster.

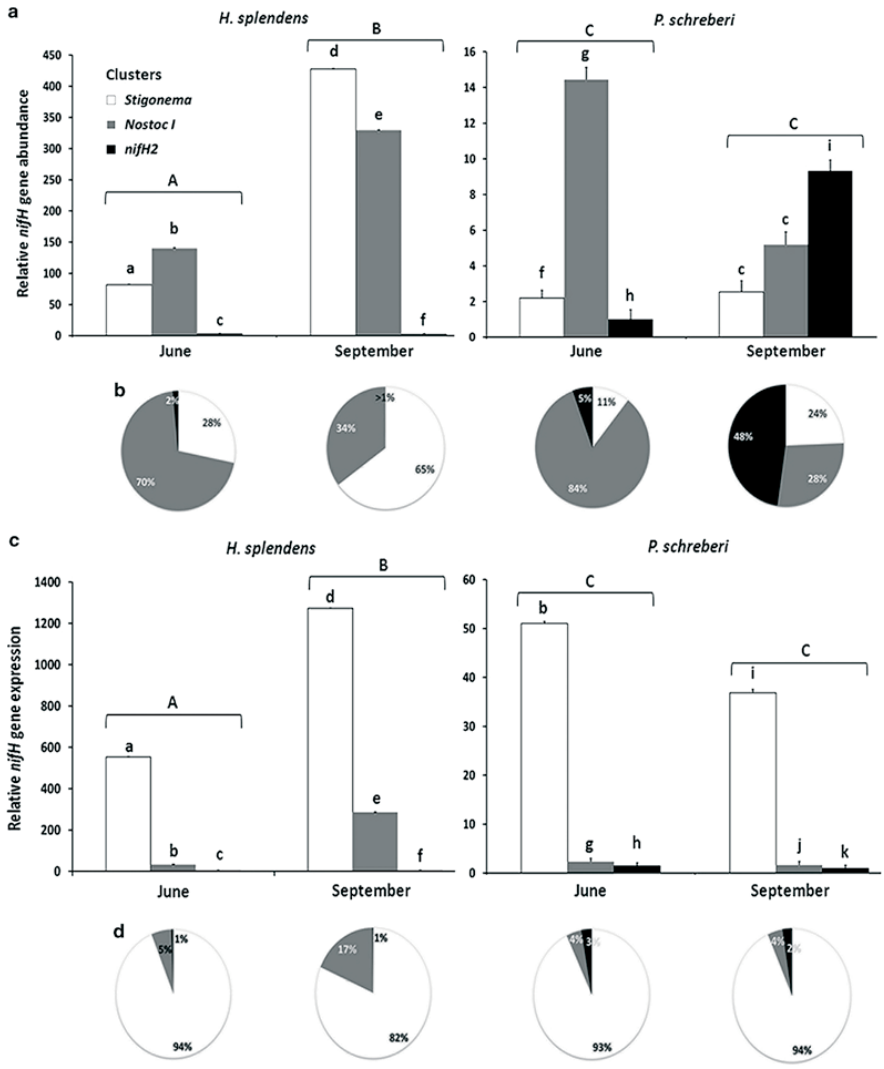


Figure 10. Average (±SE) *nifH* gene abundance and expression relative to 16S rRNA gene expression of the cyanobacterial community associated with *H. splendens* and *P. schreberi* in June *versus* September. (a) Relative *nifH* gene abundance in the cyanobacterial community. (b) *nifH* gene abundance expressed as percentage of each *nifH* cluster in the cyanobacterial community. (c) Relative *nifH* gene expression in the cyanobacterial community. (d) *nifH* gene expression as a percentage of each *nifH* cluster in the cyanobacterial community. For (a) and (c), note the difference in scale between *H. splendens* and *P. schreberi*. Significant differences between moss species × month combinations are indicated by different capital letters on the top of groups of three histogram bars, following Tukey's HSD test ($\alpha<0.05$). Different lower case letters represent significant differences between the *nifH* clusters, following Tukey's HSD test ($\alpha<0.05$). Taken from Warshan et al. (2016).

Effect of the climate change factors - temperature and CO_2 levels - on cyanobacterial N_2-fixation activities, and on moss growth rate.

The effect of elevated temperature and CO_2 levels (ETC) on the composition of the cyanobacterial communities, and if those changes affect N_2-fixation and moss growth, was investigated (**Paper IV**). The symbiosis between *P. schreberi* gametophytes and different *Nostoc* strains isolated from feather-mosses was reconstituted at different levels of diversity, with one, five and 20 *Nostoc* strains, and were further exposed to different temperatures (11°C and 19°C) and CO_2 concentrations (500 ppm and 1000 ppm). The cyanobacterial N_2-fixation rates and MGR were measured and the cyanobacterial community structure and composition were followed by targeted amplicon sequencing of the *nifH* gene.

Under the scenario of climate change with elevated temperature and CO_2 levels, N_2-fixation rates supported by *P. schreberi* grown at 19°C were on average 1.7 times higher than for the one grown at 11°C (23.5 and 38.9 μg N_2 fixed g^{-1} moss FW d^{-1} for 11°C and 19°C, respectively) (**Paper IV**). Combined elevation of temperature and CO_2 levels had a strong positive effect on the N_2-fixation rates, on average the rates were 2.4 times higher under ETC compared to the normal conditions (**Paper IV**). The effect of combined elevation of temperature and CO_2 levels on MGR was also significant, and on average the rates were 2.8 times higher at ETC compared to normal conditions (0.55 and 1.52% gain of mg FW $week^{-1}$ for 500 ppm and 1000 ppm, respectively) (**Paper IV**). Based on studies with *P. schreberi*, N_2-fixation activities have previously been reported to be affected by both temperature alone as well as interactively with factors such as light and moisture (Gentili et al., 2005; Gundale et al., 2012; Rousk et al., 2017). Further, short-term treatment (90 days) with increased CO_2 and temperature had been found to stimulate growth and N_2-fixation rates of *N. punctiforme* (Lindo and Griffith, 2017). Elevated CO_2 levels (ca. 200 ppm above ambient) were also shown to positively affect the growth rate of the moss *Sphagnum* sp. after one year of exposure, but not when the gametophytes were exposed for a longer period (Toet et al., 2006). Thus, the results obtained in this thesis might represent a short-term positive effect of elevated CO_2 levels on MGR, and effects of longer exposure (more than one year) should be investigated. In addition, structural equation modeling (SEM; Figure 11) was performed to help to disentangle the effect of ETC. The results of SEM suggest a direct positive impact on the N_2-fixation rates, and an indirect positive influence on MGR through increased N_2-fixation activities by the cyanobacterial community (Figure 11; **Paper IV**). Considering that N_2 fixed by cyanobacteria has been shown to be transferred to the moss (Bay et al., 2013), the observed increased N_2-fixation rates might lead to increased N transferred and thus promote moss growth.

The SEM results suggest that the alpha diversity was in overall reduced under ETC (**Paper IV**). Increased temperature has been shown to favor higher species richness, but also to result in the extinction of cold adapted species in certain regions such as at high elevations and latitudes (Thomas et al., 2004; Bellard et al., 2012; Pacifici et al., 2015). In this study, changes in cyanobacterial community composition by ETC were dependent on identity of the cyanobacteria strain (**Paper IV**). The growth of *N.* Moss3 cluster appeared to be favoured by ETC, on the contrary *Nostoc* cluster I, and the *Stigonema* cluster were negatively impacted (**Paper IV**). The cyanobacteria affiliated to *N.* Moss5/6 were found to be more resilient than any other cluster to ETC (**Paper IV**), suggesting a weak environmental filtering on these cyanobacteria strains.

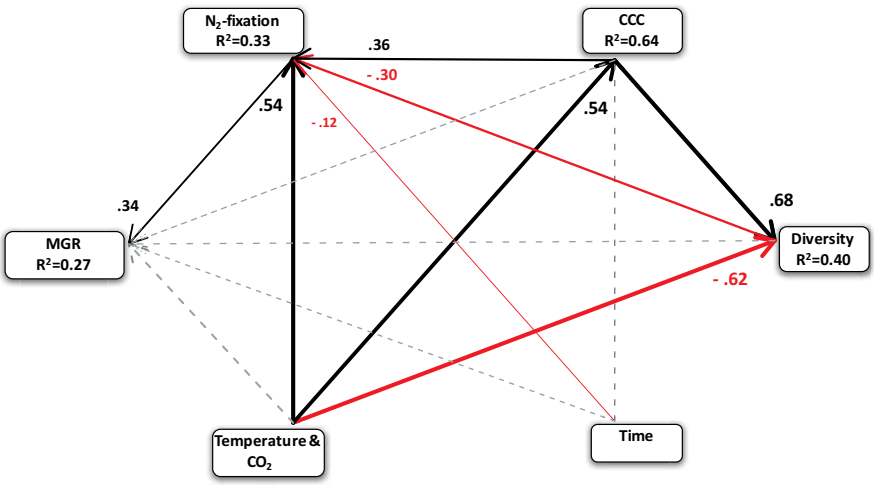


Figure 11. Structural Equation Model (SEM) showing the proposed causal relationships of the climatic condition and incubation time on the cyanobacterial community diversity and composition, and the relations of these components with N_2-fixation and MGR. Black and red lines indicate significant positive and negative pathways, respectively. Dotted lines indicate non-significant pathways. Width of the line indicates the strength of the relationships.

Organisms can be characterized according to their life-history strategy: r-strategists that have high growth rates and low resource use efficiency, and K-strategists that have low growth rates and high resource use efficiency (Klappenbach et al., 2000; Fierer et al., 2007). Consequently, cyanobacteria belonging to *Nostoc* cluster I, *N.* Moss2 and the *Stigonema* cluster might be considered as K-strategists, and display lower competitiveness under ETC compared to cyanobacteria belonging to *N.* Moss3, *N.* Moss5/6 and the mixed clusters. These cyanobacteria could in turn be considered as r-strategists, displaying stronger competitiveness and colonization abilities under ETC. There

is evidence from soil microbial communities that K-strategists are less resilient, to climate change-related disturbances than r-strategists (Haddad et al., 2008; Bapiri et al., 2010; De Vries et al., 2012; Lennon et al., 2012).

Conclusions

This thesis was trying to contribute to our understanding of cyanobacteria-plant symbioses in general and more specifically of the cyanobacterial symbiosis with the feathermoss *Pleurozium schreberi*. The analyses in this thesis were framed around general themes: the evolution of cyanobacteria in symbiosis with plants, the cyanobacterial mechanisms involved in forming symbiosis with feathermosses, the identity and role of the different cyanobacteria within the community in symbiosis with feathermosses, and the future of this partnership under climate change.

Concerning the evolutionary history of cyanobacterial symbionts, the phylogenetic reconstruction based on the concatenated alignment of proteins shows that symbiotic *Nostoc* strains with a broad host-range, including both intracellular and extracellular physical localizations, form a monophyletic clade indicating that the members of this clade have a common evolutionary history. In addition, a polyphyletic origin was found for *Nostoc* strains able to enter only extracellular symbioses, suggesting that this trait was most likely gained several times in the evolution of the Nostocales. Molecular clock analysis suggested that the intracellular competent clade emerged ~600 Mya, which predates the origin of land plants, and the origin of Gunneridae. This finding indicates that the clade of cyanobacteria capable of intracellular symbioses evolved before *Gunnera*, and this type of symbiotic lifestyle even may have predated the emergence of extant terrestrial plants.

This thesis also attempts to deepen our understanding of putative symbiotic mechanisms used by cyanobacteria to form symbioses with their plant partners. The genomic investigations revealed the gene families shared between all symbiotic cyanobacteria but also highlighted gene families differentially retained and specific to intracellular-capable strains. The genomic changes of these symbiotic *Nostoc* strains over evolutionary time resulted in an establishment of basic molecular mechanisms between cyanobacterial and plants within the genome of the lowest common ancestor of Nostocales. Embellishments on genomes of symbiotic *Nostoc* have been diverse, and resulted in multiple evolution of plant-cyanobacterial symbioses. Only a small set of genes was retained in the genomes of all facultative symbiotic *Nostoc* strains, suggesting that the trait of extracellular symbiosis could have been gained by horizontal gene transfer. When comparing the members of the two clades of extracellular-restricted *Nostoc* to the intracellular capable strains, a specific set of genes was gained during evolution that might have enabled these strains

to form an intracellular symbiosis with *Gunnera* sp. All facultative symbiotic *Nostoc* strains have enriched functions in organic sulfur transport and metabolism, chemotaxis and motility, phosphate/amino acid and ammonium uptake.

Global gene and protein expression experiments conducted on cyanobacteria in interaction with the feathermoss *P. schreberi* confirmed that most of the genes identified in the genomic survey were differentially regulated at the transcriptional and post-translational level during the establishment and maintenance of the symbiosis, but also highlighted new molecular pathways, such as NO-sensing. It was also shown that the symbiotic competent *Nostoc* strains differentially express a unique set of genes related to motility, NO regulation, and foster a distinct extracellular environment compared to the non-competent strain *Nostoc* sp. CALU 996. Investigation of gene regulations after colonization of the feathermoss highlights that the *Nostoc* symbiosis with feathermoss is distinct from endophytic-symbioses with plants, most likely due to its epiphytic nature. The differences are through the retention of motility and chemotaxis post-colonization, as well as constitutive regulation of genes related to N_2-fixation, photosynthesis, GS-GOGAT cycle, and heterocyst formation.

This thesis work underlined the role of biotic factors, namely cyanobacterial community diversity and composition, into predicting N_2-fixation activity. From field experiments, it was shown that the variation in N_2-fixation rates was greatly explained by temporal changes in *nifH* expression by different cyanobacterial *nifH* clusters, and especially by the *nifH* affiliated to the genus *Stigonema*. Although not dominating, *Stigonema* might be the most influential N_2-fixer in the cyanobacterial community and potentially cyanobacteria from this genus are responsible for the main input of N in the boreal forest ecosystems. In addition, this thesis results demonstrated that the cyanobacterial community structure and composition is vulnerable to climate change. In the scenario of a climate change with elevated temperature and CO_2 levels, it was shown that the cyanobacteria community structure and composition might be important regulators of N_2-fixation and moss growth rates under predicted future abiotic conditions. The predicted elevation of temperature and CO_2 levels might also result in the reduction of cyanobacterial diversity. Moreover, the 'new' climatic conditions can induce changes in cyanobacterial community composition resulting in the dominance of cyanobacteria adapted to the future abiotic factors. The results suggest a weak abiotic environmental filtering of key cyanobacterial strains for N_2-fixation and moss growth, and an overall increased N_2-fixation rates. The results implied a higher N input in the boreal forests by the feathermoss-cyanobacteria symbiosis in the future climatic conditions.

Future perspectives

This thesis made contributions to the knowledge in the field of cyanobacterial-plant symbioses, and more specifically on the understanding of the feathermosses-cyanobacteria symbiosis. Yet, this work generates numerous new questions and sets the ground for further hypotheses to be tested to deepen our knowledge in this topic such as:

- The phylogenomic investigations revealed that the monophyletic clade of Intra/Extra symbiotic *Nostoc* predates the origin of Gunneridae, raising the question on the identity of the original host. One can hypothesize that the ancestor of the extant symbiotic *Nostoc* develops symbiotic mechanisms with its original host, and further evolved new mechanisms to switch eukaryotic host, such as fungi and plants. This thesis includes all genomes of symbiotic *Nostoc* at present available. Thus, investigating this question by phylogenomic requires more sequencing to increase the number of available genomes of symbiotic *Nostoc* strains isolated from various plant hosts, but also from the fungus *Geosiphon pyriformis* as well as a genomic inventory of terrestrial and marine cyanolichens. The results of this thesis also highlight the fact that the intracellular symbiotic capacity may have been acquired in a single lineage, and subsequently part of the symbiotic gene repertoire may have been spread by horizontal/lateral gene transfer among the extracellular clades. To test for horizontal/lateral gene transfer, one approach can be to search for sections of a genome that significantly differ from the genomic average of all available *Nostoc* strains, such as guanine-cytosine (GC) content or codon usage. Moreover, single gene tree of each of the symbiotic candidate gene can also be used to examine their evolutionary histories and identify inconsistent phylogenies between trees.
- The results of this thesis suggest shared cyanobacterial symbiotic mechanisms between *Nostoc* strains in symbiosis with a broad range of host plants. Vice-versa, one can hypothesized that host plants from different lineages shared common signaling pathways to establish and maintain a symbiosis with cyanobacteria. By increasing the number of sequenced genomes of host plants, comparative genomics approach of closely related host and non-host plants can be performed.

This approach might help to uncover a symbiotic gene repertoire conserved in host plants capable of forming symbiosis with cyanobacteria.

- The thesis generates a set of cyanobacterial genes presumably involved in forming symbiosis with plants. Further steps to validate these candidate genes would be the functional characterization by gene knockouts to validate the role of the identified molecular pathways in the symbiosis. For instance, deletion of the H-NOX for NO-sensing in cyanobacteria could reveal if this pathway is involved in cross-talk between partners. Gene deletions, allelic replacements and introduction of mobilizable shuttle vectors in *Nostoc* can be performed following the protocol developed for *Nostoc punctiforme* in Cohen and Meeks (1997) or Liaimer et al. (2011). Recently the CRISPR technology and the alternative nuclease Cpf1 was used for gene knockout in the Nostocacean strain *Anabaena* PCC 7120, and may represent an alternative to the classical methods (Ungerer and Pakrasi, 2016). Ultimately, symbiotic reconstruction technique can be used to characterize the symbiotic phenotypes of the mutants and confirm the role of the identified pathways in plant-cyanobacteria symbioses.

- During the transcriptomic study performed in this thesis, transcriptomes of *P. schreberi*, when grown without cyanobacteria and during physical contact with *Nostoc* strains, were also generated. These transcriptomes can be used for differential gene expression to characterize putative feathermoss genes differentially regulated during symbiosis. This analysis could help to identify putative feathermoss gene set involved in the establishment of the feathermoss-cyanobacteria symbiosis. Moreover, it is known that uncharacterized compounds are secreted by the feathermoss, and act as HIF and chemo-attractant (Bay et al., 2013). We can use the moss transcriptomes for proteins mapping of the exoproteome of *P. schreberi* when in chemical contact with cyanobacteria. This approach can help to characterize the extracellular environment of the moss in the early phase of the symbiosis and may give insights on the identity of the HIF secreted by the plant. To complement the transcriptomic and proteomic approach mutagenesis of *P. schreberi* could help to characterize the function of the genes identified in the transcriptomic and proteomic analyses. This can culminate in the comparison of the symbiotic phenotype of the moss, mutant vs wild type, i.e. difference in colonization performance, host-induced hormogonia differentiation, directed-motility of the hormogonia, maintenance of the symbiosis, and N-enrichment of the moss tissue ($\delta^{15}N$ and/or SIMS).

- NO was hypothesized to be a new communication molecule employed by the feathermosses to interact with its cyanobacterial partners. To verify this hypothesis biochemical techniques such as the use of nitric-oxide specific fluorescent probe 4,5-diaminofluorescein-2 diacetate (DAF-2 DA), can help to trace the accumulation of NO in the moss tissue. This fluorescent probe can be used in complement to the NO-scavenger, c-PTIO, as well as nitrate reductase inhibitors, such as tungstate or NO synthase inhibitor L-NNA and L-NAME. Cyanobacteria and mosses can be grown separately with tungstate, L-NNA and/or L-NAME, and symbiotic reconstitution experiments on the treated samples can be performed. NO-production or accumulation by the cyanobacteria and/or the moss can be monitored and localized by the DAF-2 DA, and phenotypic traits such as colonization performance by the cyanobionts and N_2-fixation activity could be measured.

- Transcriptomic and proteomic studies on other terrestrial cyanobacterial-eukaryotes symbioses with other plants as well as fungi, can help to find out if similar molecular pathways, identified in this work, are shared and involved in establishment and maintenance of other partnership. This can be a first step towards the definition of a "cyanobacterial symbiotic gene set". Considering that many studies indicate a close relatedness between *Nostoc* genotypes of lichen species with those from plant (O'Brien et al., 2005; Stenroos et al., 2006; Myllys et al., 2007; Papaefthimiou et al., 2008a), one can hypothesized that some communalities in symbiotic mechanisms are shared between terrestrial cyanobacteria symbioses.

- Cyanobacteria belonging to the genus *Stigonema* were identified from field studies as potentially the main N_2-fixers in the cyanobacterial communities inhabiting feathermosses. Considering the presumably importance of this genus for the N input in the boreal forests, gaining insights on the genomic capabilities of *Stigonema* sp. would help to understand the physiological advantages of this genus over other symbiotic cyanobacteria. Unfortunately, this cyanobacterium was understudied, mostly because it has not been successfully grown in culture. However, one approach could be to sequence *Stigonema* genome using single cell sequencing.

- The *nifH* phylotyping of the cyanobacterial community over two growth seasons highlighted host-dependent compositional changes between seasons. However, following the compositional changes over several years might shed light on new cyanobacterial strains or other biotic interactions that were missed due to the short time scale. Complementary to an increased experimental time scale, such a study

should include monitoring of the availability of nutrients such as P, Fe, and Mo, and their effects on the cyanobacterial community composition and consequently on N_2-fixation rates and moss growth. Climate change is predicted to impact on organic matter decomposition, and subsequently could affect Mo, Fe, V availability (Rousk and Michelsen, 2016). One can hypothesize that these changes in abiotic conditions and micro-nutrients dynamics could select specific cyanobacterial strains in the community e.g. cyanobacteria with higher affinity for the uptake of these nutrients would be selected.

- Considering that members of the feathermoss-inhabiting microfauna have been recently shown to exert top-down control of N_2-fixation rates by the cyanobacteria (Kardol et al., 2016), it appears necessary to know which are the key-microfaunal species for this process and to understand how this top-down control affects the cyanobacterial community. The use of shotgun metagenomic sequencing in complement to micro-fauna isolation and identification can help to characterize the genetic and taxonomic diversity of the microfauna and microbes found in the bryosphere. Metabolic profiling based on the metagenomics sequencing can help to assess the diversity of functions that are represented in the bryosphere. Moreover, how the top-down control will change under the future climatic conditions in boreal forests needs to be understood to properly predict the fate of the cyanobacteria-feathermosses symbiosis. To investigate this question, the metagenomics investigations can be coupled by metatranscriptomic and shotgun mass spectrometry-based metaproteomics, on samples exposed to different climatic conditions, such as increased temperature. N flux i.e. transfer of N from cyanobacteria to the host, and further to the microfauna and other microbes, could be monitored by high-resolution secondary ion mass spectrometry (SIMS), in order to understand the trophic relationships in the bryopshere, and how these will be affected under changed climatic conditions. This study could help to disentangle the interactions between feathermosses, cyanobacteria and the associated microfauna and microbes, but also provide insights on how these relationships will evolved under future climate conditions.

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