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Seasonal variation in *nifH* abundance and expression of cyanobacterial communities associated with boreal feather mosses

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Dinitrogen (N_2)-fixation by cyanobacteria living in symbiosis with pleurocarpous feather mosses (for example, *Pleurozium schreberi* and *Hylocomium splendens*) represents the main pathway of biological N input into N-depleted boreal forests. Little is known about the role of the cyanobacterial community in contributing to the observed temporal variability of N_2-fixation. Using specific *nifH* primers targeting four major cyanobacterial clusters and quantitative PCR, we investigated how community composition, abundance and *nifH* expression varied by moss species and over the growing seasons. We evaluated N_2-fixation rates across nine forest sites in June and September and explored the abundance and *nifH* expression of individual cyanobacterial clusters when N_2-fixation is highest. Our results showed temporal and host-dependent variations of cyanobacterial community composition, *nifH* gene abundance and expression. N_2-fixation was higher in September than June for both moss species, explained by higher *nifH* gene expression of individual clusters rather than higher *nifH* gene abundance or differences in cyanobacterial community composition. In most cases, '*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. Stepwise multiple regressions showed temporal variations in N_2-fixation being greatly explained by variations in *nifH* expression of the '*Stigonema* cluster'. These results suggest that *Stigonema* is potentially the most influential N_2-fixer in symbiosis with boreal forest feather mosses.

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Introduction

Dinitrogen (N_2)-fixation by filamentous heterocystous cyanobacteria living in symbiosis with pleurocarpous feather mosses (for example, *Pleurozium schreberi* and *Hylocomium splendens*) has been shown to provide nitrogen (N) to their mosses (Bay *et al.*, 2013), and this serves as a major input of N into boreal forests (DeLuca *et al.*, 2002; Turetsky *et al.*, 2012; Jonsson *et al.*, 2014), and this input can be as high as 3.5 kg N ha^{-1} per year (Zackrisson *et al.*, 2004; Lindo *et al.*, 2013). Several studies have revealed that N_2-fixation by these cyanobacteria can vary greatly across stands of different successional ages (Zackrisson *et al.*, 2004; Lagerström *et al.*, 2007; Gundale *et al.*, 2010), within and between years (DeLuca *et al.*, 2007; Gundale *et al.*, 2012b), and among moss species (Leppänen *et al.*, 2013; Lindo *et al.*, 2013). *P. schreberi* and *H. splendens* often dominate different microhabitats where *P. schreberi* occupies a wider range of environments while *H. splendens* dominates in mesic and more productive sites (Cronberg *et al.*, 1997; Zackrisson *et al.*, 2009). Even when the two mosses coexist in the same microhabitat, the N_2-fixation rates have been shown to differ among the species (Zackrisson *et al.*, 2009; Gundale *et al.*, 2012a; Leppänen *et al.*, 2013; Stuiver *et al.*, 2015). The role of different extrinsic environmental factors in driving the observed spatial and temporal variability of N_2-fixation rates have been studied (Gentili *et al.*, 2005; Gundale *et al.*, 2009, 2012a, b; Jackson *et al.*, 2011; Jean *et al.*, 2012; Sorensen *et al.*, 2012), whereas little is known about the role of the cyanobacterial community contributing to this variability. A better knowledge of this issue will contribute to a more complete understanding of the mechanisms underpinning N inputs and dynamics of boreal forest. The cyanobacterial communities living in symbiosis with feather mosses have been characterized primarily through morphological observations and culture-dependent studies, and

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the association has been regarded as a promiscuous symbiotic association consisting of multiple cyanobacterial species belonging to the genera *Nostoc*, *Calothrix*, *Fischerella*, *Cylindrospermum* and *Stigonema* (Gentili *et al*., 2005; Houle *et al*., 2006; Zackrisson *et al*., 2009). In the study by Ininbergs *et al*. (2011), 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*. They showed that the cyanobacterial community is host-specific and composed of five different clusters that were represented by *nifH* phylotypes belonging to the genera previously known to live on these feather mosses. Moreover, 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.

The aim of the present study was to provide insights into 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 of the cyanobacterial community and whether this is consistent over the growing season. Specifically, we investigated, for each moss species, whether temporal variation in N_2-fixation rates is related to variation in cyanobacterial abundance and community composition. Further, for each moss species and over the growth season, we aimed to explore how N_2-fixation activity (as shown through *nifH* expression) of individual cyanobacterial clusters within the community contributes to overall N_2-fixation rates. Thus, specific *nifH* primers targeting individual cyanobacterial clusters in the community were designed and used to quantify their abundance and expression by quantitative PCR (qPCR). Firstly, we hypothesized that the cyanobacterial abundance and community composition (that is, the relative abundances of different individual clusters) differ between coexisting moss species (that is, *P. schreberi* versus *H. splendens*), and that these will change over the growing season for both species. Secondly, we hypothesized that the relative *nifH* expression of specific clusters within the cyanobacterial community varies between moss species as well as across the growing season for both moss species. Finally, we hypothesized that the majority of cyanobacterial N_2-fixation will be performed by only a few of the clusters present, and that N_2-fixation rates therefore will be well correlated with the activity of those clusters alone.

Materials and methods

Sampling sites and moss collection

The gametophytes of *P. schreberi* and *H. splendens* used in this experiment were randomly collected from nine forest stands in Northern Sweden (65°35′–65°60′N and 18°14′–19°10′E) on 15 June and 10 September 2010. These two periods correspond to the main N_2-fixation seasonal peaks of moss–cyanobacteria symbioses (DeLuca *et al*., 2002; Lagerström *et al*., 2007). In each stand, we set up three main plots (each 10 m in diameter and at a distance of 15 m from each other) along a 60 m transect. In each plot, we collected a bundle of gametophytes of both moss species from about 30 randomly chosen microspots over the whole area, and all gametophytes from within each main plot were pooled to result in three within-stand replicates. With this approach, we were able to account for within-plot variation in microhabitat properties. Sample collection was performed only further than 2 m from overstory trees and in patches that were devoid of dense shrub vegetation. All forest stands are open-canopy late post-fire successional forest stands that have not been affected by fire over the last 300 years. All stands were dominated by coniferous trees (*Pinus sylvestris* and *Picea abies*), with the understory mainly composed of ericaceous shrubs (*Vaccinium myrtillus*, *V. vitis-idaea* and *Empetrum hermaphroditum*), and a thick ground layer of feather mosses (*P. schreberi* and *H. splendens*). Similarly to other boreal regions, N deposition has been shown to be very low in this area (<2 kg ha^{-1} year; Lagerström *et al*., 2007).

N_2-fixation activity measurements

The N_2-fixation rates of each moss species (that is, *P. schreberi* and *H. splendens*) were indirectly measured by acetylene reduction assay (Hardy *et al*., 1968), for each of the three plots in each of the nine forest stands at the two sampling times (that is, June and September), making a total of 54 measurements for each moss species. For each measurement, either five *H. splendens* gametophytes or 10 *P. schreberi* gametophytes were placed in a 22-ml glass sealed vial (Gundale *et al*., 2010; Ininbergs *et al*., 2011). As *P. schreberi* gametophytes generally have half the mass of those of *H. splendens*, the amounts of material added per vial were similar for both species (that is, mean ± s.e. of 0.03 ± 0.0028 g dry weight for *P. schreberi* and 0.03 ± 0.0032 g dry weight for *H. splendens*). Only the upper green part (*ca.* 3–5 cm) of the moss gametophytes was used as this is where most of the N_2-fixation activity occurs (Solheim *et al*., 2004). After field collection but prior to N_2-fixation analysis, all samples were kept outdoors for 72 h in a natural forest site near the laboratory in Umeå, Sweden (63°49'N—20°15'E) and misted with deionized water to keep both cyanobacteria and mosses hydrated until further experimentation. Measurements of the levels of ethylene produced after 24-h incubation were performed as described by Ininbergs *et al*. (2011). A ratio of three moles of ethylene per mol of N was used to convert acetylene reduction rates to μg N fixed g^{-1} fresh weight moss d^{-1} (DeLuca *et al*., 2002; Zackrisson *et al*., 2004; Lagerström *et al*., 2007).

Diurnal N_2-fixation activity

As Ruttjeheden was the site where the highest variation in N_2-fixation rate between June and September was observed (Figure 1), the site was selected for further study of *nifH* abundance and gene expression using qPCR analysis. To identify the time of the day when the N_2-fixation rates peak and the samples for RNA isolation should be sampled, a diurnal study was conducted on *P. schreberi* and *H. splendens* collected from Ruttjeheden the day following the acetylene reduction assay measurements for the nine forest stands described above. On 17 June and 12 September, five *H. splendens* gametophytes and 10 *P. schreberi* gametophytes were placed in each of 36 vials as described above, and the vials were arranged into three independent replicate blocks. Therefore, each block consisted of 12 vials, with 6 vials for each moss species. Every 4 h, one vial from each moss species from each block was sealed and incubated with acetylene for 4 h (that is, 6 vials × 4 h incubation each = 24 h). For each vial, the measurement of N_2-fixation through acetylene reduction was performed directly after the 4 h incubation period, as described above. Each sample was then immediately snap-frozen and kept in liquid N to prevent DNA/RNA degradation until the start of extractions. All incubations with acetylene occurred in a natural forest site near the laboratory in Umeå, Sweden (63°49′N—20°15′E), where light intensity and temperature were recorded every 2 h.

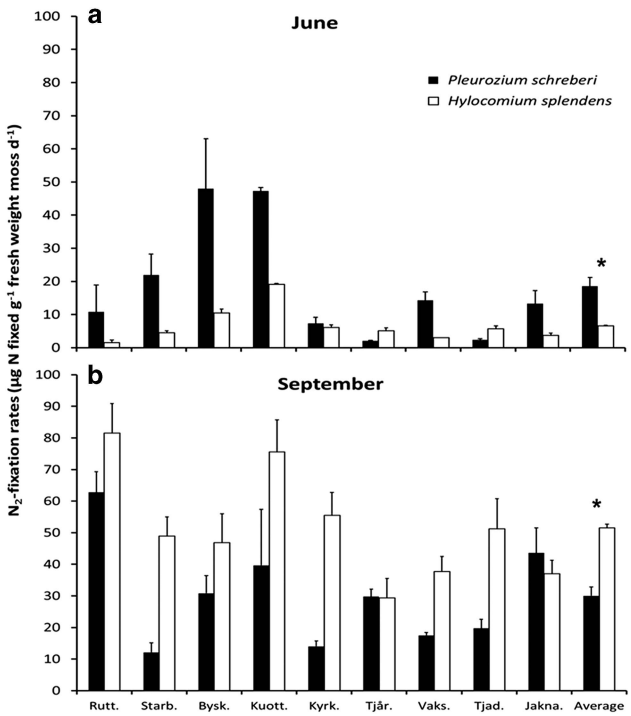

Figure 1 N_2-fixation rates (mean ± s.e.) for *P. schreberi* and *H. splendens* collected in June (**a**) and September (**b**) 2010 expressed as µg N fixed g^{-1} fresh weight moss d^{-1}, at each of nine late successional boreal forest stands in northern Sweden ($n = 3$). The 'average' presented is the mean of N_2-fixation rates measured at the nine sites with each forest stand as independent replicates ($n = 9$) for each moss species at each sampling date. ANOVA results are presented in Supplementary Table 1. Forest sites: Rutt. = Ruttjeheden, Starb. = Starbetjvare, Bysk. = Byskeälven, Kuott. = Kuottavare, Tjår. = Tjårre, Vaks. = Vaksliden, Tjad. = Tjadnes, Jakna. = Jaknapuoda. *indicates significant differences between average N_2-fixation rates measured on each moss species ($\alpha < 0.05$).

DNA/RNA extractions

As the diurnal study revealed that the highest N_2-fixation activity occurred between 16:00 and 20:00 in June for both moss species, and between 08:00 and 12:00 in September for both moss species (Figure 2), frozen moss samples corresponding to those times of the day (three replicates for each moss species) were used for qPCR analysis. Moss samples were homogenized in liquid N and approximately 250 mg of each sample were further processed for DNA/RNA co-purification using the NucleoSpin RNA Plant and NucleoSpin RNA/DNA Buffer Set (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations, but with slight modifications to improve cell lysis using the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA). For DNA samples, the yield was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For RNA samples, genomic DNA contamination was removed using the Ambion DNA-free kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations. After DNase treatment, the total amount of RNA was quantified with a NanoDrop 1000 spectrophotometer. Complementary DNA was generated from 100 ng of RNA pooled samples using the qScript Flex cDNA Kit (Quanta Biosciences Inc., Gaithersburg, MD, USA), following the manufacturer's instructions for gene-specific priming of the cyanobacterial *nifH* gene (Olson *et al*., 1998) and the housekeeping gene coding for cyanobacterial 16S rRNA (Nübel *et al*., 1997). We used DNA, cDNA and negative controls (minus-RT and water) as templates for quantitative PCR assays.

Primer design

On the basis of the alignment of *nifH* sequences carried out by Ininbergs *et al*. (2011), specific primers targeting each cyanobacterial cluster were designed (that is, '*Nostoc* cluster I', '*Nostoc* cluster II', '*Stigonema* cluster' and '*nifH*2 cluster', respectively; Table 1). Primer sequences were subjected to BLASTN (Altschul *et al*., 1997) to confirm that they were targeting each cyanobacterial cluster in the GenBank database. To reconfirm the specificity of the individual primer sets, the qPCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Minden, Germany) and sequenced using the respective forward primer from each product. The retrieved sequences were included in a phylogenetic analysis using the same nucleotide sequences as Ininbergs *et al*. (2011). Nucleotide sequences were aligned in Ugene (Okonechnikov *et al*., 2012), using the MUSCLE algorithm and

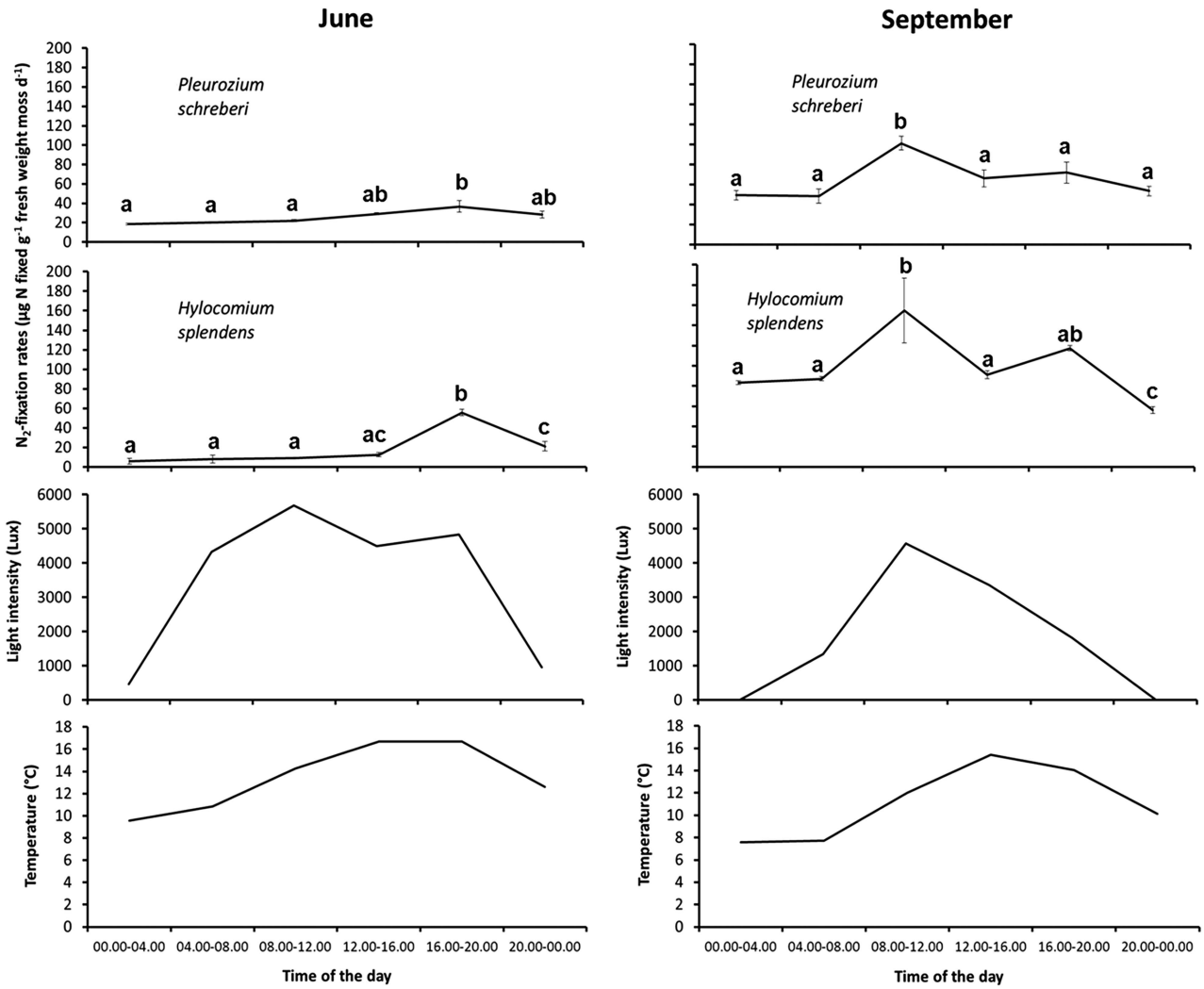

Figure 2 Diurnal variation of cyanobacterial N_2-fixation rates (mean ± s.e.) for *P. schreberi* and *H. splendens* sampled from Ruttjeheden in June and September, and of light and temperature at the incubation site. Data are obtained after consecutive acetylene reduction assay measurements performed after 4 h incubations. In each panel, different letters above data points indicate statistically significant differences between N_2-fixation rates measured at different times of the day for each moss species at each sampling date (ANOVA followed by Tukey's HSD test, $\alpha < 0.05$). ANOVA results are presented in Supplementary Table 2.

Table 1 Primers used to amplify cyanobacterial DNA and cDNA in this study

Primer	*Sequence (5′ → 3′)*	*References*
Ncl I-F	CACCGTTCTTCACCTCGCC	This study
Ncl I-R	TGCATACATCGCCATCATTTC	This study
Stig-F	TTCACCTCGCTGCTGAACGTGG	This study
Stig-R	TCCAAGAAGTTGATAGCGGTAAT	This study
Ncl II-F	GCTGACCGGTTTCCGGGATGT	This study
Ncl II-R	CAACATCTTTGTAAGCACCG	This study
*nifH*2-F	AACCCGGTGTTGGTTGCGCT	This study
*nifH*2-R	ATGGCCATCATCTCGCCGGA	This study
nifH cyano-R	GCATACATCGCCATCATTTCACC	Olson *et al*., 1998
16S cyano-F	CGGACGGGTGAGTAACGCGTGA	Nübel *et al*., 1997
16S cyano-R	GACTACTGGGGTATCTAATCCCATT	Nübel *et al*., 1997

Abbreviations: Ncl I, *Nostoc* cluster I; Ncl II, *Nostoc* cluster II; *nifH*2, *nifH*2 cluster; Stig, *Stigonema* cluster.

a phylogenetic dendrogram was inferred. Branch support was obtained with 100 bootstraps replicates.

Cyanobacterial 16S rRNA primer pair (Nübel *et al*., 1997) was evaluated using the TestPrime web tool (http://www.arb-silva.de/search/testprime). Primer specificity and coverage for the most commonly observed cyanobacteria genera on *P. schreberi* and *H. splendens* (*Nostoc*, *Calothrix*, *Fischerella*, *Cylindrospermum* and *Stigonema*) and cross-reactivity with non-cyanobacteria sequences was determined with ARB software package (Ludwig *et al*., 2004) using the SILVA RefNR SSU-123 sequences (Quast *et al*., 2013).

Quantitative PCR and quantitative reverse transcription PCR

All qPCR analyses were carried out in a mixture of 20 µl containing 0.3 µM of each primer, 10 µl of SYBR Green, 1 µl of master mix (Roche Diagnostics Scandinavia AB, Bromma, Sweden), 4 mM $MgCl_2$ (Qiagen) and 3% DMSO (Thermo Scientific) using LightCycler 480 (Roche Diagnostics Scandinavia AB). Four microliters of DNA (dilution 1/5) and cDNA

(dilution 1/10) were used as template. The PCR conditions comprised a denaturation step of 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 64–68 °C for 40 s and no extension step to avoid non-specific amplification. Annealing temperatures for the different cyanobacterial clusters were optimized empirically. No amplifications were detected from reverse transcriptase-free control reactions or from reactions including water instead of cDNA. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal was derived from specific PCR products and not from primer-dimers or other artifacts. Each qPCR analysis was performed in three technical replicates and additionally repeated on two independent plates. Fold changes in gene abundance and expression were calculated by the comparative $2^{-\Delta\Delta CT}$ method, using the housekeeping gene coding for cyanobacterial 16S rRNA as the normalizer for *nifH* gene abundance and expression; both were corrected for the PCR efficiency (Livak and Schmittgen, 2001) using LightCycler 480 software release 1.5.1 (Roche).

Statistical analysis

A repeated measures analysis of variance (ANOVA) was performed to test for the effects of moss species, forest site, sampling date (as the repeated measures factor) and their interactions on N_2-fixation rates. Repeated measure ANOVA was also used to test for the effects of time of the day, moss species, sampling date (as the repeated measures factor) and their interactions on N_2-fixation. Tukey's HSD *post hoc* tests were then used to help identify the time of the day at which N_2-fixation rates are highest.

To determine the transcriptional efficiency of each individual cyanobacterial cluster, we calculated the ratio between the *nifH* expression and abundance (defined as the *nifH* transcriptional efficiency of the individual cyanobacterial clusters in the population). Repeated measure split-plot ANOVAs were used to test for the effects of moss species (as the main plot factor), individual cyanobacterial cluster (as the subplot factor) and sampling date (as the repeated measure factor), and their interactions, on *nifH* gene abundance, *nifH* gene expression and *nifH* transcriptional efficiency. Whenever ANOVAs indicated that significant differences were present, Tukey's HSD *post hoc* tests were performed to compare treatment means.

Correlation analysis using Spearman's rho and stepwise multiple regressions with Akaike Information Criterion (AICc) were performed to identify the combination of variables that best predicted N_2-fixation rates. We determined which combination of variables (out of sampling date, light intensity, temperature, moss species and *nifH* expression and abundance for each individual cluster) served as best predictors of N_2-fixation rates measured in the diurnal study.

Results

Temporal variation in N_2-fixation rates of P. schreberi *and* H. splendens

The average N_2-fixation rate was approximately three times higher in September than in June (Figure 1). Moreover, the N_2-fixation rate for *P. schreberi* in June was 2.8 times higher than that of *H. splendens,* while in September, the fixation rate of *H. splendens* was 1.7 times higher than that of *P. schreberi* (Figure 1). Our ANOVA results showed that sampling date, forest site and all possible interactions involving these two factors and moss species significantly influence N_2-fixation rates (Supplementary Table 1).

Our results from the diurnal N_2-fixation measurements showed that the N_2-fixation rates in June were highest between 16.00 and 20.00 for both moss species, while in September, the highest N_2-fixation rate occurred between 08.00 and 12.00 (Figure 2). The ANOVA results showed that sampling date, the time of the day and all possible interactions involving these two factors and moss species significantly influence N_2-fixation rates (Supplementary Table 2).

Validation of cluster-specific nifH primers and cyanobacterial 16S rRNA primers

The results from the phylogenetic tree computed with the same nucleotide sequences used to design the primers and sequences obtained by qPCR revealed that all amplified qPCR products belong to their respective clusters (data not shown). Primers targeting '*Nostoc* cluster II' did not give any amplification products, indicating that cyanobacteria belonging to this cluster were not present or were below the level of detection.

In silico testing of the 16S rRNA primer pair using Silva-TestPrime with one allowed mismatch indicated an average of 93.1% coverage for the targeted cyanobacteria genera. Specifically for the two most abundant genera *Stigonema* and *Nostoc*, the primers have 100% and 96.4% coverage, respectively. In addition, the 16S rRNA primers cover 0.1% of non-cyanobacterial sequences belonging to the Synergistetes phylum.

Temporal change of the cyanobacterial community composition

The abundance of *nifH* genes on *H. splendens* was overall 28 times higher than on *P. schreberi* and 3.3 times higher on *H. splendens* in September compared with June (Figures 3a and b). Further, the community composition changed considerably between June and September for both *H. splendens* and *P. schreberi* (Figures 3a and b). In June, the '*Nostoc* cluster I' was the dominant cluster on both *H. splendens* and *P. schreberi*, and represented 70% and 84% of the total cyanobacterial community, respectively. However, in September, the '*Stigonema* cluster' became the most abundant cluster on *H. splendens,* representing 65% of the total cyanobacterial community, while the '*nifH*2 cluster' (which includes the

cyanobacterial genera *Nostoc*, *Calothrix* and *Fischerella*) became the most abundant cluster on *P. schreberi,* constituting 48% of the total community. Repeated measure ANOVA showed that *nifH* gene abundance was influenced by sampling date, moss species and cyanobacterial

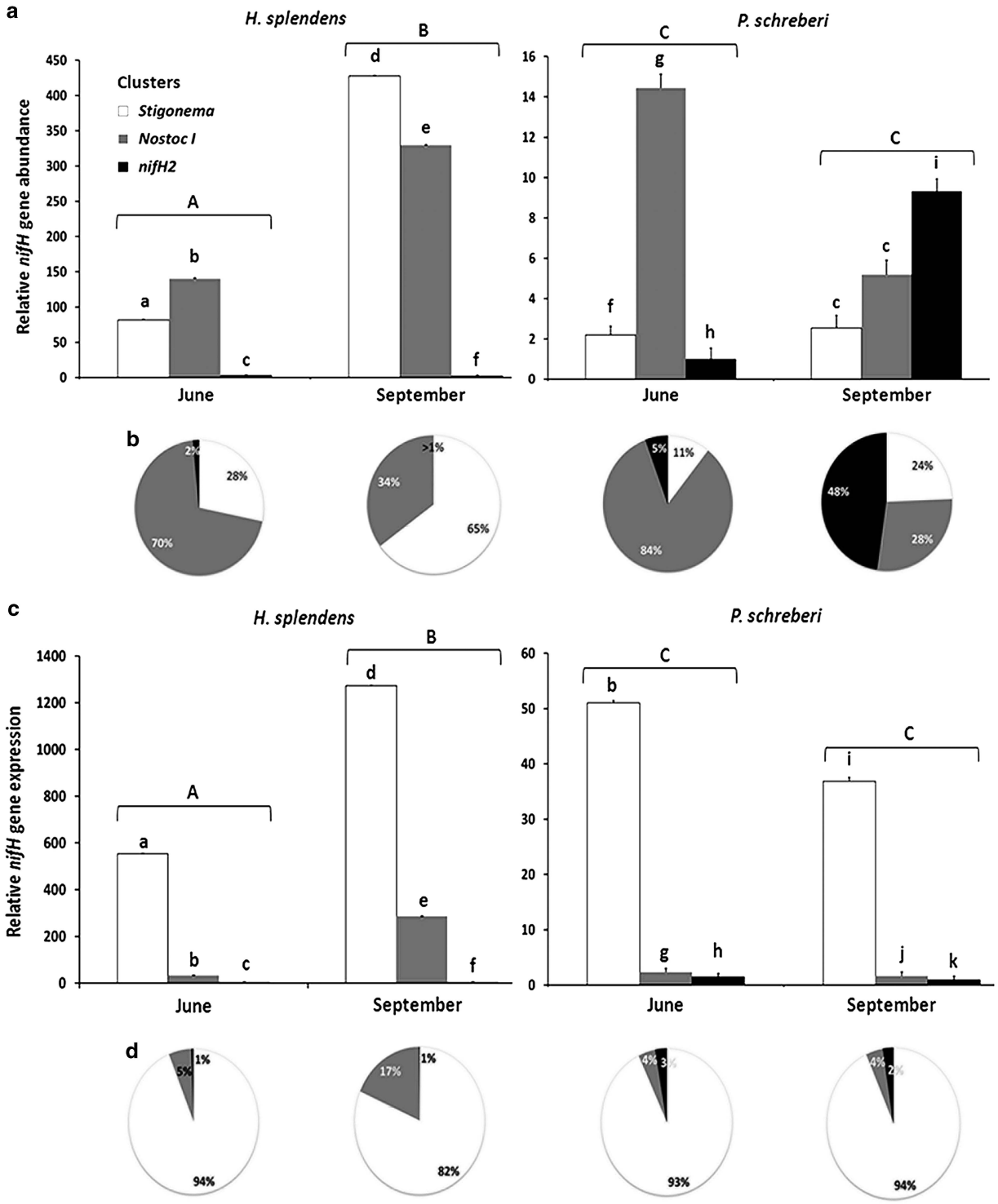

Figure 3 Average (± s.e.) *nifH* gene abundance and expression relative to the 16S rRNA gene of the cyanobacterial community associated with *H. splendens* and *P. schreberi* in June versus September. (**a**) Relative *nifH* gene abundance in the cyanobacterial community. (**b**) *nifH* gene abundance expressed as a 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 ($\alpha < 0.05$). ANOVA results are given in Supplementary Table 3.

cluster, as well as all but one of the possible interactions among these factors (Supplementary Table 3).

nifH expression of individual cyanobacterial clusters

Our result shows that the *nifH* expression of the different cyanobacterial clusters increased between June and September for *H. splendens* and remained unchanged for *P. schreberi* (Figures 3c and d). The '*Stigonema* cluster' had the highest *nifH* expression on both *H. splendens* and *P. schreberi* for both sampling dates, constituting 82–94% of the total *nifH* expression in the community, while '*Nostoc* cluster I' and '*nifH*2 cluster' had the second and third highest *nifH* gene expression, respectively (Figures 3c and d). When comparing *nifH* gene abundance (Figures 3a and b) and *nifH* expression (Figures 3c and d), it is apparent that although the '*Stigonema* cluster' was not the most abundant (with exception of *H. splendens* in September), it accounts for the majority of the *nifH* gene expression, irrespective of moss species and sampling date. Conversely, '*Nostoc* cluster I' was dominating on both moss species in June but its *nifH* expression only accounted for 4–5% of the total *nifH* expression in the community. Our results from repeated measure ANOVA revealed that community *nifH* expression was influenced by sampling date, moss species and cyanobacterial cluster, as well as all possible interactions among these factors (Supplementary Table 3).

The '*Stigonema* cluster' was the most transcriptionally efficient cyanobacterial cluster on *P. schreberi* in both sampling dates. For *H. splendens*, both '*Stigonema* cluster' and '*nifH*2 cluster' are equally efficient in June, while '*nifH*2 cluster' was the most efficient cyanobacterial cluster in September (Figure 4). Repeated measure ANOVA revealed that *nifH* transcriptional efficiency was influenced by moss species and *nifH* cluster, and all except one of the possible interactions involving these two factors and sampling date (Supplementary Table 3).

Parameters influencing the temporal dynamics of N_2-fixation rates

The correlation analysis showed that N_2-fixation rates were positively correlated with sampling date (June versus September), *nifH* gene abundance of '*Stigonema* cluster' and '*nifH*2 cluster', *nifH* gene expression of '*Stigonema* cluster' and '*Nostoc* cluster I', and light intensity (Table 2). Stepwise multiple regression analysis using data from the diurnal study showed that N_2-fixation rates could be best predicted by a model incorporating three variables, that is, *nifH* expression of '*Stigonema* cluster', sampling date and *nifH* abundance of '*nifH*2 cluster ($R^2 = 0.969$, $P < 0.001$). '*Stigonema* cluster' *nifH* expression and sampling date explained most of the variation observed for the dependent variable (51% and 33%, respectively).

Discussion

Diazotrophs associated with pleurocarpous feather mosses have a key role for 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 growing seasons (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, 2012a, b; Jackson *et al*., 2011; Jean *et al*., 2012; Sorensen *et al*., 2012; Rousk *et al*., 2013). N_2-fixation rates also differ between feather moss species (Lagerström *et al*., 2007) and these fluctuations

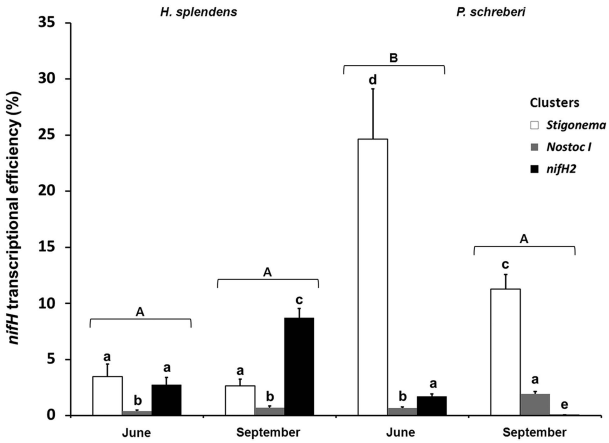

Figure 4 Average (±s.e.) *nifH* gene transcriptional efficiency (expressed as the ratio between the *nifH* expression and abundance) of the cyanobacterial clusters associated with *H. splendens* and *P. schreberi* in June and September. Significant differences between moss species × month combinations are indicated by different capital letters on 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 ($\alpha < 0.05$). ANOVA results are given in Supplementary Table 3.

Table 2 Results from Spearman correlation analysis between N_2-fixation measured at the highest peak of the day and sampling date, moss species, *nifH* expression and abundance for each individual cluster, light intensity and temperature

Factors	*N_2-fixation (μg N g^{-1} fresh weight moss d^{-1})*
Sampling date	**0.871 (0.000)**
Moss species	−0.375 (0.071)
nifH abundance of '*Stigonema* cluster'	**0.667 (0.000)**
nifH abundance of '*Nostoc* cluster I'	0.282 (0.182)
nifH abundance of '*nifH*2 cluster'	**0.449 (0.028)**
nifH expression of '*Stigonema* cluster'	**0.514 (0.010)**
nifH expression of '*Nostoc* cluster I'	**0.472 (0.020)**
nifH expression of '*nifH*2 cluster'	0.284 (0.178)
Light intensity	**0.826 (0.000)**
Temperature	−0.176 (0.411)

Values show the correlation coefficient and the *P*-value in parentheses; bold values show statistically significant correlations at $P < 0.05$.

are in part related to differing composition and diversity of cyanobacteria on these mosses (Ininbergs *et al*., 2011). With the use of specific *nifH* primers targeting four major cyanobacterial clusters in the cyanobacterial community and qPCR, we showed that the temporal variation of N_2-fixation rates is mostly determined by the *nifH* gene expression of cyanobacteria belonging to the '*Stigonema* cluster'.

In support of our first hypothesis, our data showed that the abundance and community composition of the cyanobacteria inhabiting *H. splendens* and *P. schreberi* differed, and that the cyanobacterial composition changed over the growing season for both species. 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 with that of *P. schreberi*. Therefore, these results not only provide evidence that composition is host-specific (Ininbergs *et al.*, 2011), but also suggest a promiscuous symbiosis with a mixed community changing over time. As the changes in the community composition are different for the two moss species, the drivers of cyanobacterial community composition might be directed by the host species and/or by different abiotic conditions provided by the host. Under dry conditions, the cyanobacterial community associated with *P. schreberi* was shown to fix more N than the community on *H. splendens,* suggesting that different hosts provide different abiotic conditions (Gundale *et al*., 2012a). In addition, it was recently shown that the host controls the degree of colonization by secretion of chemo-attractants when depleted in N (Bay *et al.*, 2013). A host control mechanism by *P. schreberi* could explain why we found no variation in cyanobacterial abundance over the growing season, contrary to the abundance increase observed for *H. splendens*. However, we cannot exclude the possibility that our observations could be explained in part by potential antagonistic or mutually beneficial interactions between the members of the epiphytic microbial communities in the manner that has been shown for microbial communities in the phyllosphere (Delmotte *et al.*, 2009; Dulla and Lindow, 2009; Vorholt 2012).

In support of our second hypothesis, we found that the *nifH* expressions of individual cyanobacterial clusters changed temporally for both moss species and were higher on *H. splendens* than on *P. schreberi*. Although '*Stigonema* cluster' showed the highest *nifH* expression on both moss species in both June and September, it only dominated the cyanobacterial community on *H. splendens* in September. Although *Stigonema* has been observed in association with both *H. splendens* and *P. schreberi* in previous studies (Gentili *et al*., 2005; Houle *et al*., 2006; Ininbergs *et al.*, 2011), this is the first study to show that the '*Stigonema* cluster', despite its overall relatively low occurrence in the community, is the main contributor to N input into the boreal forest. These findings are indicative that *Stigonema* is a far more important genus for N input than previously thought. The dominance of '*Stigonema* cluster' on *H. splendens* as seen in this study has also been observed in a previous study from the boreal forest of Quebec, Canada; *Stigonema* sp. was the most abundant genus on *H. splendens* compared with *P. schreberi* during the late autumn season, that is, September—October (Houle *et al*., 2006). In addition, a higher number of phylotypes affiliated to the '*Stigonema* cluster' was also observed on *H. splendens* compared with *P. schreberi* in the work of Ininbergs *et al*. (2011). It should be noted that the 16S rRNA primers used for normalizing the *nifH* abundance and expression could potentially cross-react with the non-cyanobacteria community, for example, co-occurring bacteria and archaea. Therefore, one should be cautious in interpreting the differences between samples because the co-occurring non-target community likely differs in abundance. However, *in sillico* study of the 16S rRNA primers showed a high coverage and specificity for the common cyanobacterial genera found on *P. schreberi* and *H. splendens* and a low coverage with non-cyanobacteria sequences and therefore amplification of non-target bacteria was negligible.

Both feather mosses, and especially *H. splendens*, show distinct seasonal growth patterns (Busby *et al.*, 1978; Callaghan *et al.*, 1978; Campioli *et al.*, 2009). These studies have inferred that abiotic environmental factors, such as high rainfall frequency or low temperature, are merely responsible for their biomass accumulation over time. However, none of these earlier works have recognized that these mosses host cyanobacteria that are capable of transferring N to the host (Bay *et al*., 2013). Rainfall frequency is a strong determinant of annual growth increment, carbon fixation (Campioli *et al*., 2009) and N_2-fixation rates (Jackson *et al*., 2011) of feather mosses, meaning that seasonal rainfall patterns also may have indirect effects on moss growth. Interestingly, this is also the period when the relative mass increment of the youngest (current year+1 year) segments of *H. splendens* has been reported to be the highest (Campioli *et al*., 2009). The relative growth rate and shoot biomass of *P. schreberi* also increase from early to late season, but this increase is less marked than that for *H. splendens* (Campioli *et al.*, 2009). This pattern is also reflected in our findings where the relative increase in N_2-fixation rate from June to September is lower than that of *H. splendens* and that *nifH* gene expression of cyanobacteria associated with *P. schreberi* is lower than that of *H. splendens*.

Consistent with our third hypothesis, we found that only a few clusters are responsible for the majority of N being fixed by the cyanobacterial community, in particular, the '*Stigonema* cluster', which explained most of the temporal variation for N_2-fixation. '*Stigonema* cluster' is the most transcriptionally efficient cyanobacterial cluster (relative to its abundance), especially on *P. schreberi* in June when it only represents 11% of the total *nifH* gene abundance but contributes to more than 90% of

the total *nifH* gene expression. We can potentially explain this observation by the fact that *P. schreberi* might provide a more optimal habitat to its cyanobacteria (Gundale *et al.*, 2012a). However, the *nifH* gene expressions of individual cyanobacterial clusters retrieved on *P. schreberi* in September are surprisingly low considering the difference in N_2-fixation rates observed between June and September. One explanation might be the existence in our samples of an untargeted cyanobacterial cluster or other diazotrophs highly capable of N_2-fixation. In addition, N_2-fixation activity was observed in the dark period in September, and this suggests that there may also be other diazotrophs present. As such, it was recently reported that Alphaproteobacteria are more abundant than cyanobacteria in the microbial communities associated with *Sphagnum* mosses (Bragina *et al.*, 2011). However, as Nostocaceae are capable of N_2-fixation in darkness both when free-living (Whitton *et al.*, 1979; Huber, 1986) and in symbiosis with plants (Rai *et al.*, 2002), the existence of an untargeted cyanobacterial cluster cannot be excluded.

We found a positive correlation of the N_2-fixation rates with the *nifH* expression of '*Stigonema* cluster' and '*Nostoc* cluster I'. Nevertheless, *nifH* expression of '*Nostoc* cluster I' poorly explains the N_2-fixation rate variations compared with the *nifH* expression of '*Stigonema* cluster'. In addition, the N_2-fixation rates were correlated with the *nifH* gene abundance of the '*Stigonema* cluster' and the '*nifH*2 cluster'. The dominance of *Nostoc* species (represented in the '*Nostoc* cluster I' and '*nifH2* cluster') in the cyanobacterial community is evident. However, their contribution to the *nifH* gene expression pool is minor. As a result, *Nostoc* species could most likely be considered as a 'cheater' with regards to N input into boreal forests. It is known from other symbiotic systems that hosts can select specific members of the N_2-fixing community. For instance, soybean (*Glycine max*) selects the 'cooperative strains' of *Bradyrhizobium japonicum* from a mixed community using mechanisms of sanctions/impositions and directed reciprocation (Trivers, 1971; Crespi, 2001; Kiers *et al.*, 2003). A bryophytic mechanism of sanction to control the cyanobacterial community could result from the secretion of antimicrobial cationic peptides or oxylipin molecules; these compounds have been found to be synthesized by mosses (Matsui, 2006; Skripnikov *et al.*, 2011). Finally, light intensity was the only abiotic factor found correlated with N_2-fixation, and in spite of having an important role in this metabolically costly process (Staal *et al.*, 2002; Rabouille *et al.*, 2006), it was shown to be of secondary importance in predicting N_2-fixation rate variations.

In conclusion our results demonstrate a temporal and host-dependent dynamic of the cyanobacterial communities (that is, composition, *nifH* abundance and *nifH* expression) in symbiosis with *H. splendens* and *P. schreberi*. Variation in N_2-fixation rates are greatly explained by temporal changes in cyanobacterial *nifH* expression, especially of the genus *Stigonema*, which implies that this cyanobacterial genus might—although not dominating—be the most influential N_2-fixer in this ecosystem. On the contrary, *Nostoc* proves to be the most abundant cyanobacterial genus but shows poor *nifH* transcriptional activity and could then be considered as a 'cheater' in the cyanobacterial community. Consequently, our findings not only provide new and valuable insights into N_2-fixation rate variations but also highlight the lack of knowledge about factors controlling the maintenance, composition and activity of the moss–cyanobacteria symbiosis and its eventual consequences for N input into boreal forests. Studying the role of the host and potential mechanisms regulating the cyanobacterial community appears an essential step towards a deeper understanding of the spatial and temporal dynamics of N_2-fixation in boreal forest ecosystems.

Conflict of Interest

The authors declare no conflict of interest.

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