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The moss traits that rule cyanobacterial colonization

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• Background and Aims Cyanobacteria associated with mosses represent a main nitrogen (N) source in pristine, high-latitude and -altitude ecosystems due to their ability to fix N₂. However, despite progress made regarding moss–cyanobacteria associations, the factors driving the large interspecific variation in N₂ fixation activity between moss species remain elusive. The aim of the study was to identify the traits of mosses that determine cyanobacterial colonization and thus N₂ fixation activity.

• Methods Four moss species varying in N₂ fixation activity were used to assess cyanobacterial abundance and activity to correlate it with moss traits (morphological, chemical, water-balance traits) for each species.

• Key Results Moss hydration rate was one of the pivotal traits, explaining 56 and 38 % of the variation in N₂ fixation and cyanobacterial colonization, respectively, and was linked to morphological traits of the moss species. Higher abundance of cyanobacteria was found on shoots with smaller leaves, and with a high frequency of leaves. High phenol concentration inhibited N₂ fixation but not colonization. These traits driving interspecific variation in cyanobacterial colonization, however, are also affected by the environment, and lead to intraspecific variation. Approximately 24 % of paraphyllia, filamentous appendages on Hylcomium splendens stems, were colonized by cyanobacteria.

• Conclusions Our findings show that interspecific variations in moss traits drive differences in cyanobacterial colonization and thus, N₂ fixation activity among moss species. The key traits identified here that control moss-associated N₂ fixation and cyanobacterial colonization could lead to improved predictions of N₂ fixation in different moss species as a function of their morphology.

Key words: Bryophytes, moss colony, Cyanobacteria, nitrogen fixation, water retention, functional trait.

INTRODUCTION

Nitrogen (N) fixation performed by moss-associated cyanobacteria is a main N source in many pristine ecosystems, such as boreal forests and subarctic tundra, where N deposition is low and plant growth is commonly limited by N availability (LeBauer and Treseder, 2008; Wang et al., 2010). Given the high abundance of mosses in these ecosystems, the association contributes significantly to ecosystems’ N cycle by accounting for up to 50 % of the total N input (e.g. Rousk and Michelsen, 2017). Wide-ranging taxa of mosses have been found to host cyanobacteria, but N₂ fixation activity of the association varies greatly among moss species (Rousk et al., 2015; Stuart et al., 2021). For instance, N₂ fixation activity in Hylcomium splendens can be more than double the activity found in Pleurozium schreberi, although they are co-dominant in boreal forests (Stuart et al., 2021), while the activity in H. splendens is only one-sixth the activity found in Sphagnum in arctic tundra (Rousk et al., 2015). To date, although many factors, such as nutrient availability (Gundale et al., 2011; Rousk et al., 2017) and moisture content (Rousk et al., 2015, 2018) affect N₂ fixation in mosses, we do not know why these large variations in N₂ fixation activity between mosses growing in the same habitat occur. This lack of understanding of the seemingly random colonization patterns among moss species hampers efforts to upscale N₂ fixation across moss species at a larger scale.

Cyanobacteria are the dominant N₂ fixer associated with mosses (Leppänen et al., 2013), and N₂ fixation activity is commonly correlated with the number of epiphytic cyanobacterial cells on mosses (Rousk et al., 2013a). Thus, differences in the abundance of epiphytic cyanobacteria likely drive the interspecific variation of moss-associated N₂ fixation rates. Cyanobacterial abundance, on the other hand, is affected by different factors, such as the capability to move or disperse, and the environmental factors affecting these processes (Solheim and Zielke, 2002). Among these factors, traits of the moss host, which provide microsites for epiphytic cyanobacteria (Dalton and Chatfield, 1985), might play a key role as moss traits vary greatly among species (Niinemets and Tobias, 2019), and certain moss species seem to be especially colonized by cyanobacteria (Solheim et al., 1996). However, to date, it remains unknown whether moss traits affect the colonization of cyanobacteria and, if so, which suite of moss traits facilitates cyanobacterial colonization that leads to differences between moss
hosts. The paucity of data on the effects of species-specific moss traits on cyanobacterial colonization limits our understanding of the relationship between moss and cyanobacteria, and thereby of the functioning of moss-dominated ecosystems such as boreal forests.

Studies have repeatedly found that moisture promotes N₂ fixation in moss–cyanobacteria associations (Smith, 1984; Jackson et al., 2011; Gundale et al., 2012; Rousk et al., 2018). This could be the result of either a direct positive effect of water availability on cyanobacterial activity, or indirectly, promoting the colonization of cyanobacteria, or a combination of both. To form an effective N₂-fixing association with plants, vegetative cyanobacterial cells differentiate into motile and short-lived hormogonia with a gliding activity for 48–72 h (Meeks and Elhai, 2002). The capacity to move would affect the number of cyanobacteria colonizing mosses (Sanetti et al., 2013). A moist surface or liquid is required for hormogonia to glide or swim (Brahamsha and Bhaya, 2014), and it is likely that passive water flow carries cyanobacteria from lower parts of a moss shoot to its upper segments (Broady, 1979). Studies concerning the mobility of hormogonia are mainly focused on structures of cyanobacteria (e.g. Adams and Duggan, 2008; Wilde and Mullineaux, 2015), while the characteristics of the moss host may affect movement capacity and hence the abundance of epiphytic cyanobacteria. If moss colonies absorb water at different rates, the rate of water flow may result in differences in cyanobacterial abundance between moss species and thereby differences in N₂ fixation.

Water retention capacity and water absorption rate of mosses may be important for hosting cyanobacteria, and are affected by colony structure (e.g. density and height) as well as by traits of individual shoots (e.g. leaf frequency). Indeed, moss colony density and height have been reported to correlate positively with water retention capacity (Proctor, 1982; Elumeva et al., 2011), shoot morphology controls dehydration rate of mosses (Cruz de Carvalho et al., 2019), and leaf width is positively related to water retention in Sphagnum mosses (Bengtsson et al., 2020). However, to our knowledge, there is no empirical evidence that these hydrology-related traits affect cyanobacterial colonization of mosses. Moreover, moss traits might affect colonization rates directly. Mosses are assumed to host and protect cyanobacteria between or on their leaves (Dalton and Chatfield, 1985), which vary 54-fold in size and 28-fold in frequency among species (Niinemets and Tobias, 2019), and cyanobacterial filaments are found between moss stems and leaves (Solheim and Zielke, 2002). Thus, moss species with larger leaves and higher leaf frequency should host more cyanobacteria due to higher availability of colonization sites. Yet again, the relationships between colony- (e.g. frequency of shoots and colony height), shoot- (e.g. frequency of leaves and shoot length) and leaf-level (e.g. leaf width and area) traits and cyanobacterial colonization remain elusive.

Another possible mechanism that regulates cyanobacterial colonization is the production of chemical compounds by the host. Mosses are known to produce and accumulate inhibitory compounds like phenols (Erickson and Miksche, 1974). These compounds can lead to inhibition of bacterial growth (Rousk et al., 2013a) and contributes to the low decomposability of moss litter (Lang et al., 2009). Differences in phenol concentration of host mosses might lead to variation in cyanobacterial colonization and activity. Similarly, pH is an influential factor structuring microbial communities (Rousk et al., 2009) and affecting N₂ fixation rates in mosses (Alvarenga and Rousk, 2021). To date, it is unknown if differences in the chemical environment among moss species lead to variations in the number of epiphytic cyanobacteria.

The purpose of the study reported herein was to identify the moss traits that affect cyanobacterial colonization, and thus N₂ fixation activity, using four different moss species that have been shown to vary in N₂ fixation. To accomplish this, we measured acetylene reduction rate as a measure of N₂ fixation activity, and assessed cyanobacterial colonization and abundance at shoot and colony (group of shoots) level and linked this to water balance traits (maximum water content, water absorption rate and water loss rate of moss colonies), chemical traits (pH, total phenols), colony structural traits (frequency of shoots and height) and morphological traits (shoot length, frequency of leaves, leaf area, etc., Table 1) at shoot as well as at leaf level of the four moss species collected in the subarctic region. We hypothesized that (1) N₂ fixation activity is correlated with cyanobacterial colonization in all investigated moss species, (2) the moss species that has the highest water absorption rate hosts the most cyanobacteria, (3) moss traits (e.g. frequency of shoots) that facilitate water absorption increase cyanobacterial colonization, (4) moss shoots with higher frequency of leaves host more cyanobacterial colonizers, and (5) high phenol concentration and low pH inhibit cyanobacterial colonization.

MATERIALS AND METHODS

Moss sampling

Moss samples were collected at two sites in Northern Sweden. Aulacomnium turgidum (Wahlenb.) Schwägr., Hylocomium splendens (Hedw.) Schimp. and Tomentypnum nitens (Hedw.) Loeske (Supplementary Data Figure S1) were collected in June 2019 in a subarctic dry heath close to the Abisko Scientific Research Station (68°19′02″N, 18°50′04″E). The mean annual temperature in Abisko is 0.2 °C, and the mean annual precipitation is 337 mm (30-year mean 1986–2015, Abisko Scientific Research Station 2016). The site was dominated by mosses and Vaccinium uliginosum, Andromeda polifolia and Rhododendron lapponicum (see Rousk and Michelsen, 2017). Pleurozium schreberi (Brid.) Mitt., which did not occur at this site, was collected in August 2019 in a boreal forest near Arvidsjaur (64°58′49″N 19°33′59″E). This extended the study to another species that has been shown previously to differ in N₂ fixation rates compared to H. splendens despite their similar morphology and habitat preferences (Jean et al., 2020). Mean annual temperature and precipitation are approximately 1 °C and 570 mm respectively. The forest was dominated by Picea abies, V. vitis-idaea, V. myrtillus and Empetrum hermaphroditum (see Rousk et al., 2013b). From this site, samples of H. splendens were also collected in order to identify differences in the measured variables across ecosystems, as well as to ascertain if differences in N₂ fixation is a species or an ecosystem effect.
Three separate monospecific moss colonies, with at least 5 m distance from each other, were selected for each species at the subarctic and boreal sites. Uniform moss colonies of 15 cm × 15 cm were sampled and carefully transported to the laboratory in Copenhagen. The samples were assessed for \( \text{N}_2 \) fixation activity, water balance (maximum water content, water absorption and -loss rate), chemical (pH and total phenols), shoot and leaf morphological traits, and cyanobacterial counts. The \( \text{H. splendens} \) samples from the boreal forest site were assessed for \( \text{N}_2 \) fixation activity, water balance, and chemical traits.

**Morphological traits and cyanobacterial colonization**

Three shoots from each sample were randomly selected to measure shoot and leaf morphological traits (\( n = 9 \) per species). We measured the length of each shoot after submerging in double distilled (dd) \( \text{H}_2\text{O} \) to ensure full hydration. For \( \text{H. splendens} \), shoots were divided into three segments according to innate growth markers. These segments were: top-most current year segment which are younger than one-year (ca. 11 mm), the segment younger than 2 years but older than 1 year, and the segment older than 2 years. The stem and six random branches were chosen from each segment for leaf measurements and cyanobacterial counting. For \( \text{P. schreberi} \), and \( \text{T. nitens} \), six random branches were chosen from each shoot. For \( \text{A. turgidum} \), which does not have branches, only stems leaves were measured. Stems of \( \text{A. turgidum} , \text{P. schreberi} \) and \( \text{T. nitens} \) were divided into two sections: the top sections, approximately 9 mm for \( \text{A. turgidum} \), 13 mm for \( \text{P. schreberi} \) and 8 mm for \( \text{T. nitens} \), which represents 1 year’s length growth (Bauer et al., 2007), and other sections. Brown segments that were partly decomposed were not included in the measurements. Fully hydrated leaves from these chosen branches and stem sections (351 branches/stem sections in total: 18 for \( \text{A. turgidum} , 72 \) for \( \text{P. schreberi} , 72 \) for \( \text{T. nitens} \) and 189 for \( \text{H. splendens} \)) were measured.

To be able to link morphological traits to cyanobacterial colonization, we destructively harvested all the leaves from 1- to 3-mm lengths of stem segments or branches through scraping to enable counting. The number of scraped-off leaves was counted using an Olympus SXZ16 stereo microscope. In total, 4857 leaves (328 for \( \text{A. turgidum} \), 1141 for \( \text{P. schreberi} \), 1469 for \( \text{T. nitens} \), and 1919 for \( \text{H. splendens} \)) were scraped off. The number of cyanobacteria-colonized leaves among scraped-off leaves was counted using an Olympus BX61 ultraviolet-fluorescence microscope with a green filter. The frequency of colonized leaves for each stem segment or branch was calculated according to the number of colonized leaves and the number of all scraped-off leaves. Digital images of five randomly selected leaves were taken with a USB2.0 CMOS Camera (ToupTek, Hangzhou, China) attached to the stereo microscope. From these images, the maximum width, basal width, length and area of individual leaves (Table 1) were measured with ImageJ 2.35 (Wayne Rasbund, National Institutes of Health, Bethesda, MD, USA). The cyanobacterial cells on the five selected leaves per stem segment or branch were counted using the Olympus BX61 ultraviolet-fluorescence microscope. The leaf size and number of cyanobacterial cells for 1755 leaves (90 for \( \text{A. turgidum} \), 360 for \( \text{P. schreberi} \), 360 for \( \text{T. nitens} \) and 945 for \( \text{H. splendens} \)) were measured. Microscopic counting, rather than using proxies of

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>AR</td>
<td>Acetylene reduction rate</td>
<td>mmol g(^{-1}) DW h(^{-1})</td>
</tr>
<tr>
<td>( F_{\text{L,Colonized}} )</td>
<td>Frequency of cyanobacteria colonized leaves</td>
<td>%</td>
</tr>
<tr>
<td>( \text{CC L,St} )</td>
<td>Cyanobacteria count on stem leaves</td>
<td>Cells leaf(^{-1})</td>
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<tr>
<td>( \text{CC L,Br} )</td>
<td>Cyanobacteria count on branch leaves</td>
<td>Cells leaf(^{-1})</td>
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<tr>
<td>( \text{CD L,St} )</td>
<td>Cyanobacteria density on stem leaves</td>
<td>Cells mm(^{-2}) leaf</td>
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<tr>
<td>( \text{CD L,Br} )</td>
<td>Cyanobacteria density on branch leaves</td>
<td>Cells mm(^{-2}) leaf</td>
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<tr>
<td>( \text{WC Max} )</td>
<td>Maximum water content</td>
<td>min</td>
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<tr>
<td>( \text{W Absorb} )</td>
<td>Time needed for moss colony to absorb water from air dried status to 50 % of maximum water content. Larger number means lower hydration rate.</td>
<td>h</td>
</tr>
<tr>
<td>( \text{W Lose} )</td>
<td>Time needed for moss colony to lose water from 100 to 50 % of maximum water content. Larger number means lower desiccation rate.</td>
<td>h</td>
</tr>
<tr>
<td>( H_{\text{ colony}} )</td>
<td>Height of moss colony</td>
<td>mm</td>
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<tr>
<td>( \text{F}_{\text{L,Shoot}} )</td>
<td>Frequency of Shoot</td>
<td>Shoots cm(^{-2})</td>
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<tr>
<td>( \text{pH} )</td>
<td>pH</td>
<td>-</td>
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<tr>
<td>( \text{Phenol} )</td>
<td>Total phenol concentration</td>
<td>mg GAE g(^{-1})</td>
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<tr>
<td>( [\text{C}] )</td>
<td>Carbon concentration</td>
<td>%</td>
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<tr>
<td>( [\text{N}] )</td>
<td>Nitrogen concentration</td>
<td>%</td>
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<tr>
<td>( L_{\text{sh}} )</td>
<td>Length of shoot</td>
<td>mm</td>
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<tr>
<td>( \text{F}_{\text{L,Colonized}} )</td>
<td>Frequency of cyanobacteria colonized leaves</td>
<td>%</td>
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<tr>
<td>( \text{W}_{\text{L,Base,St}} )</td>
<td>Basal width of stem leaf</td>
<td>mm</td>
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<td>( \text{W}_{\text{L,Max,St}} )</td>
<td>Maximum width of stem leaf</td>
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<td>( A_{\text{L,St}} )</td>
<td>Area of stem leaf</td>
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<td>( \text{W}_{\text{L,Base,Br}} )</td>
<td>Basal width of branch leaf</td>
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<td>Area of branch leaf</td>
<td>mm(^2)</td>
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cyanobacterial quantity (Renaudin et al., 2021), enabled us to link leaf traits with cyanobacterial colonization.

Water balance

Water absorption. To measure water absorption rates of moss colonies, the bottom of transparent polypropylene cups, 3.7 cm in diameter, were cut off and replaced with cotton mesh (Supplementary Data Fig. S2). We placed a round moss colony, which fits the area of the cup, into the cup. Moss patches had similar height to those in the field, and included both green and basal parts, while decomposed parts were excluded.

Cups filled with moss colonies were air-dried in a ventilated growth chamber for one week. The air temperature in the chamber was 6 °C in the night (1800–0600 h) and 12 °C during daytime (0600–1800 h). The relative humidity in the chamber varied between 51.2 and 97.7 %, with an average of 76.6 %. The cups were then weighed and placed into a plastic box, which was filled with ddH2O to 1 cm depth. The cups were weighed at time intervals of 2 min for the initial 20 min, then weighed at intervals of 5 min for the rest of the first hour, and then weighed every hour until their mass became nearly constant. We added ddH2O to the box during the experiment to maintain the same water level throughout the measurements.

Water loss. The same cups filled with moss colonies were used to determine the water loss rate of the moss colonies. Water (ddH2O) was added to the box to immerse the moss colonies and left for 12 h, allowing the colonies to reach full hydration. The cups were then placed on a tilted plastic surface for 5 min to let the surplus water run down. The samples were weighed and kept in the same chamber as above and allowed to dry. The cups were weighed every hour during the initial 12 h and every 6 h for a maximum of 120 h until their mass became nearly constant. Then we counted the number of moss shoots in each cup, oven-dried the mosses at 65 °C for 48 h and recorded their dry weight.

Calculations. Maximum water content of moss colonies were calculated and expressed as percentage of dry weight (Table 1). Exponential functions weight = K/(1 + exp(a + b × time)) and weight = a × exp(−b × time) were fitted to the moss weight and time data in the water absorption and loss experiment, respectively. The parameters, K, a, and b, were calculated using the “nls(“) function in R. The exponential functions closely imitate the moss weight changes through time during water absorption and loss processes (Supplementary Data Fig. S2). We used principal component analyses (PCAs) to identify the main traits driving the variation in cyanobacterial colonization and N2 fixation rate. (ARA). For this, 20-mL glass vials containing ten fully hydrated moss shoots (n = 3 for each species) were sealed and 10 % of the headspace was replaced with acetylene. The moss samples were incubated for 10 h at 12 °C, 10 h at 6 °C, then 4 h at 12 °C. Ethylene generated in the headspace by the cyanobacterial nitrogenase enzyme was measured by gas chromatography with a flame ionization detector using an automatic headspace sampler (Agilent, 8890 GC System, Agilent, Santa Clara, USA). The fresh moss shoots were oven-dried at 65 °C for 48 h and ground into fine powder, which was subsequently used for total carbon (TC), total nitrogen (TN) and phenol concentration measurement.

Nutrient concentrations, total phenols and pH measurements

Carbon and N concentrations in moss tissue were assessed with a Vario Macro Cube Elemental Analyzer (Elementar, Germany). Total phenols were measured in moss tissue that had been ground into fine powder and then suspended in 10 mL ethanol. Samples were shaken for 120 min and then centrifuged at 3600 g for 10 min. The supernatant was analysed for phenols using the Folin–Ciocalteu reagent. The absorbance was measured at 725 nm using a spectrophotometer. The pH of mosses was measured in 3 g fresh moss tissue that was submerged in 15 mL ddH2O and then shaken for 60 min. The pH of the extracts was determined with a pH electrode.

Statistical analyses

We first performed principal component analyses (PCAs) using acetylene reduction rate and cyanobacterial colonization variables, as well as the water balance, chemical and morphological traits to obtain an overview of the multidimensional cyanobacterial colonization and moss traits spectrum of variation. Because of close relationships of cyanobacterial count, density and morphological traits of branch leaves to those of stem leaves, only variables and traits of stem leaves were included in the analyses. The relationships between acetylene reduction rate and frequency of colonized leaves were tested with linear regression analyses. We used linear regressions to identify the main traits driving the variation in cyanobacterial colonization and N2 fixation rate: (1) relationships of water balance traits (maximum water content, hydration and desiccation rate) with cyanobacterial activity and colonization; (2) the effects of colony trait (frequency of shoots), shoot and leaf traits (shoot length and leaf width) on hydration rate and cyanobacterial colonization; (3) the relationships between cyanobacterial colonization and shoot as well as leaf traits (frequency of leaves, leaf length maximum and basal leaf width, and leaf area); and (4) the effects of chemical traits (pH and phenols) on N2 fixation activity and cyanobacterial colonization.

Differences in N2 fixation activity across moss species were compared with one-way ANOVA. Species-specific differences in cyanobacterial colonization were compared by linear mixed models, in which species identity was included as a fixed effect and colony and shoot were included as random effects. The ANOVA and linear mixed models were followed by Tukey’s HSD test. Variation in frequency of colonized leaves across
different sections of moss shoots as well as across moss species was compared by two-way ANOVA. Differences in $N_2$ fixation activity, colony structure and chemical traits of $H. \text{splendens}$ between sites were tested with t-tests. To demonstrate the variance pattern of moss traits, intra- and inter-specific coefficients of variation (CVs) of water balance, colony, chemical, shoot and leaf morphological traits were calculated. The differences in CV between intra- and inter-species were compared by Krishnamoorthy and Lee’s modified signed-likelihood ratio test (Marwick and Krishnamoorthy, 2019). Acetylene reduction rate, leaf cyanobacterial count and cyanobacterial density were log10-transformed before all analyses. All analyses were conducted with R v.3.6.1 (R Core Team, 2019), and all tests were considered significant when $P < 0.05$.

**RESULTS**

Moss species differences in $N_2$ fixation activity

Large variations were found in $N_2$ fixation activity and cyanobacterial colonization between different species ($F_{3,14} = 12.10$, $P < 0.001$, Table 2). The highest $N_2$ fixation activity was found in $H. \text{splendens}$ (113.00 ± 67.18 nmol g$^{-1}$ DW h$^{-1}$), while the lowest $N_2$ fixation was found in $P. \text{schreberi}$ (0.69 ± 0.23 nmol g$^{-1}$ DW h$^{-1}$). The $N_2$ fixation activities of $T. \text{nitens}$ and $A. \text{turgidum}$ were 42.02 ± 30.12 and 12.19 ± 3.93 nmol g$^{-1}$ DW h$^{-1}$, respectively. Accordingly, a higher frequency of $H. \text{splendens}$ leaves (54.19 ± 9.36 %) was colonized compared with $T. \text{nitens}$, $A. \text{turgidum}$ and $P. \text{schreberi}$ leaves (19.14 ± 5.24, 34.54 ± 6.25 and 18.19 ± 2.76 %, respectively).

Covariation of $N_2$ fixation activity, cyanobacterial colonization and moss traits

The PCA for $N_2$ fixation activity and cyanobacterial colonization revealed one major principal component axis, which explained 81 % of the overall variance (Fig. 1A), suggesting a strong covariation of $N_2$ fixation activity and cyanobacterial colonization. Confirming the patterns in the PCA, we found significant and positive correlations among $N_2$ fixation activities and frequency of colonized leaves ($F_{3,24} = 10.27$, $P < 0.001$) and density at shoot level ($W_{\text{Absorb}}$, $F_{3,14} = 9.27$, $P = 0.001$, Fig. 3) were significantly different among species. $H. \text{splendens}$, with the fastest hydration rate, had the highest cyanobacterial colonization frequency and $N_2$ fixation activity.

Regression analysis revealed a strong negative relationship between time for 50 % water absorption ($W_{\text{Absorb}}$) and $N_2$ fixation activity (i.e. positive relation between hydration rate and $N_2$ fixation activity), and the moss colony hydration rate explained 56 % of the variation in $N_2$ fixation activity ($R^2 = 0.56$, $P < 0.001$, Fig. 4). A similar relationship was found between hydration rate and cyanobacterial colonization, for $W_{\text{Absorb}}$ explained 38 % variation in frequency of colonized leaves ($F_{3,24} = 0.38$, $P = 0.034$, Fig. 4).

There was no significant relationship between shoot frequency ($F_{\text{Sh}}$) and hydration rate. But shoot length ($L_{\text{Sh}}$) and basal width of stem leaves ($W_{\text{L,Base,S}}$) explained 42 % ($P = 0.024$) and 43 % ($P = 0.020$) variation in hydration rate ($W_{\text{Absorb}}$), respectively (Fig. 5).

**Cyanobacterial colonization and hydration rate**

Significant differences in frequency of colonized leaves ($F_{3,24} = 3.07$, $P < 0.001$) were found among species. Maximum water content ($W_{\text{Max}}$, $F_{3,14} = 16.17$, $P < 0.001$), hydration rate (time for 50 % water absorption, $W_{\text{Absorb}}$, $F_{3,14} = 10.27$, $P < 0.001$) and decicassion rate (time for 50 % water loss, $W_{\text{Deci}}$, $F_{3,14} = 9.27$, $P = 0.001$, Fig. 3) were significantly different among species. ($H. \text{splendens}$, with the fastest hydration rate, had the highest cyanobacterial colonization frequency and $N_2$ fixation activity.

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**Links between cyanobacterial colonization and morphological traits**

The longer the moss shoot ($L_{\text{Sh}}$), the fewer leaves were colonized at shoot level ($F_{3,24} = 0.38$, $P = 0.033$, Supplementary Data Fig. S5). Similarly, shoot length was negatively correlated with cyanobacterial count (branch leaves, $R^2 = 0.21$, $P = 0.020$) and density at shoot level (stem leaves, $R^2 = 0.38$, $P = 0.001$) variation in hydration rate ($W_{\text{Absorb}}$).

**Table 2. Mean values and s.e. (n = 3) of acetylene reduction rates (AR, nmol g$^{-1}$ DW h$^{-1}$), frequency of colonized leaves ($F_{\text{L,Colonized}}$ %), cyanobacteria count on stem leaf ($CC_{\text{L,St}}$, cells per leaf) and branch leaf ($CC_{\text{L,Br}}$, cells per leaf) and cyanobacteria density on stem leaf ($CD_{\text{L,St}}$, cells mm$^{-2}$ leaf area) and branch leaf ($CD_{\text{L,Br}}$, cells mm$^{-2}$ leaf area), and their differences among species. Differences in AR between species were tested with one-way ANOVA. Differences in cyanobacterial colonization were tested with a linear mixed model in which species was included as a fixed factor and colony and shoot were included as random effects.**

<table>
<thead>
<tr>
<th>Species</th>
<th>AR (3.93)</th>
<th>CC_{L,St} (5.39)</th>
<th>CC_{L,Br} (3.72)</th>
<th>CD_{L,St} (5.03)</th>
<th>CD_{L,Br} (3.72)</th>
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<tr>
<td><em>Aulacomnium turgidum</em></td>
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<td>23.20</td>
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<tr>
<td><em>Hylocomium splendens</em></td>
<td>113.00</td>
<td>34.54</td>
<td>12.19</td>
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<tr>
<td><em>Pleurozium schreberi</em></td>
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<tr>
<td><em>Tomentypnum nitens</em></td>
<td>28.95</td>
<td>23.20</td>
<td>7.12</td>
<td>26.63</td>
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</table>

**Table 2. Mean values and s.e. (n = 3) of acetylene reduction rates (AR, nmol g$^{-1}$ DW h$^{-1}$), frequency of colonized leaves ($F_{\text{L,Colonized}}$ %), cyanobacteria count on stem leaf ($CC_{\text{L,St}}$, cells per leaf) and branch leaf ($CC_{\text{L,Br}}$, cells per leaf) and cyanobacteria density on stem leaf ($CD_{\text{L,St}}$, cells mm$^{-2}$ leaf area) and branch leaf ($CD_{\text{L,Br}}$, cells mm$^{-2}$ leaf area), and their differences among species. Differences in AR between species were tested with one-way ANOVA. Differences in cyanobacterial colonization were tested with a linear mixed model in which species was included as a fixed factor and colony and shoot were included as random effects.**

<table>
<thead>
<tr>
<th>Species</th>
<th>AR (3.93)</th>
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</table>
A higher frequency of leaves was found at leaf level (Supplementary Data Fig. S7). Fewer leaves were colonized ($F_{L,Colonized}$) with increasing leaf basal width ($W_{L,Base}$; $R^2 = 0.56$, $P = 0.005$, Fig. 5). Cyanobacterial count (CC) and density (CD) were negatively related to all assessed leaf size traits (leaf length, $L_{leaff}$; leaf basal width, $W_{L,Base}$; leaf maximum width, $W_{L,Max}$; and leaf area, $A_l$) at colony level (Fig. 6). Similar negative correlations were also found at leaf level (Supplementary Data Fig. S7).

Cyanobacterial densities of branch leaves (CD$_{Br}$) and stem leaves (CD$_{St}$) showed positive correlations with frequency of leaves ($F_{L}$; branch leaves, $R^2 = 0.77$, $P = 0.002$; stem leaves, $R^2 = 0.36$, $P = 0.041$, Fig. 6). Shoot-level analyses revealed similar positive relations between frequency of leaves ($F_{L}$) and cyanobacterial colonization (CC$_{Br}$, CD$_{L,St}$, CD$_{L,Br}$ and $F_{L,Colonized}$) (Supplementary Data Fig. S5).

There was no significant correlation between pH and colonization rates of paraphylla.

Ecosystem differences in $H. splendens$ colonies and colonization rates of paraphylla

No significant differences were found in N$_2$ fixation activities in $H. splendens$ between the two ecosystems (arctic tundra versus boreal forest), although the mean N$_2$ fixation activity of $H. splendens$ colonies from the tundra site was over 7 times higher than that of $H. splendens$ colonies from the forest site (Fig. 8). However, $H. splendens$ colonies from the tundra site were characterized by a higher shoot frequency ($F_{L,Colonized} = 0.016$), lower colony height ($H_{Colonized}$, $P = 0.015$) and lower desiccation rate (longer time for 50% desiccation, $W_{Lose}$, $P = 0.006$, Fig. 8) than colonies from the forest site.

Stems of $H. splendens$ are covered by filamentous appendages, paraphylla. Cyanobacteria colonized 19.2 and 30.0% of paraphyllia on the youngest, top sections and older sections, respectively (Fig. 9). The frequency of paraphylla colonized by cyanobacteria was significantly lower than that of leaves on the respective shoot sections ($F_{1,31} = 10.47$, $P = 0.003$).
Fig. 3 Differences in acetylene reduction (AR), frequency of colonized leaves (FL,Colonized), maximum water content (WC Max ), time for 50 % water absorption (W Absorb, hydration rate) and time for 50 % water loss (W Lose, desiccation rate) among moss species. Larger W Absorb and W Lose indicate lower hydration rate and desiccation rate, respectively. Different lower case letters above error bars indicate significant (P < 0.05) differences among species according to Tukey’s HSD test. Letters on x-axes are acronyms of studied species, i.e. At, Aulacomnium turgidum; Hs, Hylocomium splendens; Ps, Pleurozium schreberi; Tn, Tomentypnum nitens.

R² = 0.33, P = 0.01
R² = 0.56, P < 0.01
R² = 0.21, P < 0.06
R² = 0.05, P = 0.47
R² = 0.38, P = 0.03
R² = 0.17, P = 0.19

Fig. 4 Acetylene reduction rate (AR, nmol g⁻¹ dw h⁻¹) and frequency of colonized leaves (FL,Colonized) in relation to water balance traits, maximum water content (WC Max ), time for 50 % water absorption (W Absorb, hydration rate) and time for 50 % water loss (W Lose, desiccation rate). Each coloured dot represents one moss colony grouped by species identity indicated with different colours and ecosystem types, arctic tundra (filled circles) and boreal forest (filled triangles). The grey shading around the regression lines represent 95 % confidence intervals of the fitted values.
DISCUSSION
This study presents the first demonstration of the linkage between cyanobacterial colonization and traits of the moss hosts, while the moss could be simply used as a substrate by colonizing cyanobacteria. Further, our data supports our first hypothesis (H1), stating that $N_2$ fixation activity in moss–cyanobacteria associations is closely related to the abundance of epiphytic cyanobacteria. A strong link between cyanobacterial abundance and $N_2$ fixation activity has been found in previous studies (e.g. Smith, 1984; Rousk et al., 2013a). This allows an interchangeable use of activity and abundance as well as extrapolation from one to the other, easing large-scale experiments.

Linking hydration rate and cyanobacterial colonization
Our results reveal that hydration rate of the moss host, not water content per se, controls cyanobacterial colonization (Fig. 4), corroborating our second hypothesis (H2), that the moss species with the highest hydration rate hosts the most cyanobacteria. Cyanobacteria can only move in liquid or on moist surfaces (Brahamsha and Bhaya, 2014) and their mobility is transient (Bay et al., 2013). Hence, higher hydration rates can promote cyanobacterial colonization of moss leaves via passive transport along the moss shoot. This notion could also explain Bay et al. (2013)’s finding that Polytrichum commune induces hormogonia, the cyanobacterial infectious units, but does not host cyanobacteria. This could be attributed to the endohydric nature of the moss in which, compared with ectohydric mosses, water is conducted internally and the surface of the moss is water-repellent (Proctor, 2000), thereby preventing cyanobacterial colonization via external water flow along the moss shoot. Hence, our findings could explain moisture’s positive effect on $N_2$ fixation in moss–cyanobacteria associations found in earlier studies (e.g. Gundale et al., 2012; Rousk et al., 2018).

Our results that moss colonies comprising longer shoots and with wider leaves take more time to reach 50 % hydration (Fig. 5) confirmed the morphological control over hydration of moss colonies (Larson, 1981). We found morphological traits (shoot length and leaf width) related negatively to cyanobacterial colonization, and conversely, traits that facilitate hydration increase cyanobacterial colonization, supporting our third hypothesis (H3). Shoot length was negatively correlated with cyanobacterial count and density (Supplementary Data Fig. S5). This could be the result of lower hydration rates of colonies comprising longer shoots, or the longer distances the cyanobacteria need to move to colonize longer shoots, or a combination of both. Confirming the shoot length effect, we found a lower frequency of colonized leaves at the top 1- to 2-cm section than that for other sections (Supplementary Data Fig. S6).

Fig. 5 Time for 50 % water absorption ($W_{\text{Absorb}}$, hydration rate) and frequency of colonized leaves ($F_{\text{Colonized}}$) in relation to frequency of moss shoots ($F_{\text{Sh}}$), shoot length ($L_{\text{Sh}}$), and basal width of stem leaves ($W_{\text{L,Base,St}}$). Each coloured dot represents one colony grouped by species identity indicated with different colours. $W_{\text{Absorb}}$ and $F_{\text{Sh}}$ were measured at colony level, and $F_{\text{Colonized}}$, $L_{\text{Sh}}$, and $W_{\text{L,Base,St}}$ represent the mean value of three shoots. The grey shading around the regression lines represent 95 % confidence interval of the fitted values.
Leaf width was negatively correlated with hydration rate, and thus negatively correlated with colonization (Fig. 5). Although wider leaves were found to hold more water by enabling leaves to curve in *Sphagnum* species (Såstad and Flatberg, 1993; Bengtsson et al., 2020), the increase in leaf width results in a lower number of leaves per unit shoot length (Niinemets and Tobias, 2019), which reduces water-holding capacity and hydration rate at the shoot level.

**Leaf size and number trade-off, and the effect on cyanobacterial colonization**

Surprisingly, all measured traits related to leaf size (area, width etc.) were negatively correlated with cyanobacterial colonization, probably related to desiccation rates of larger, broader leaves, while leaf frequency was positively related to colonization (Fig. 6), supporting our fourth hypothesis (H4), that moss shoots with higher frequency of leaves host more cyanobacteria.

The negative relationship between leaf size and colonization is likely driven by a trade-off between leaf size and number. Leaf size is negatively correlated to leaf number in both vascular plants (Kleiman and Aarssen, 2007) and mosses (Niinemets and Tobias, 2019). We found a strong negative correlation between leaf frequency ($F_L$) and area ($A_L$) (Supplementary Data Fig. S4), confirming the leaf size versus number trade-off. The trade-off implies that the smaller the leaf size, the more leaves per unit stem length the plant can carry. Mosses with up to 55 leaves mm$^{-1}$ shoot length fall at the extreme end of the size versus number trade-off (Niinemets and Tobias, 2019). In *H. splendens* collected from the tundra, we found a range of 52–115 leaves mm$^{-1}$ shoot, which is much higher than leaf frequencies from the study above, and an area of 0.23 ± 0.01 mm$^2$ for branch leaves and 0.48 ± 0.04 mm$^2$ for stem leaves. In correspondence to the extremely high leaf frequency, *H. splendens*...
hosts the most cyanobacteria among the studied mosses (Fig. 6), and has the highest N$_2$ fixation activity (Figs 2 and 3). On the other end of the trade-off spectrum, *P. schreberi* has the largest leaves, with an area of 0.70 ± 0.05 mm$^2$ for branch leaves and 1.41 ± 0.07 mm$^2$ for stem leaves, and a low leaf frequency (33.90 ± 3.78 leaves mm$^{-1}$ shoot) among the three pleurocarpous mosses. *Pleurozium schreberi* was the least colonized by cyanobacteria and had the lowest N$_2$ fixation activity. *Tomentypnum nitens* has slightly smaller leaves (0.64 ± 0.02 mm$^2$ for branch leaves and 1.26 ± 0.09 mm$^2$ for stem leaves) than *P. schreberi* and a similar leaf frequency of 33.35 ± 4.24 leaves mm$^{-1}$ shoot length. Accordingly, *T. nitens* has a slightly higher cyanobacterial colonization rate than *P. schreberi*. Although the non-branching acrocarp, *A. turgidum*, has the largest leaf area (1.68 ± 0.07 mm$^2$) and lowest leaf frequency (9.12 ± 0.98 leaves mm$^{-1}$ shoot length) among the studied mosses, colonization rate and N$_2$ fixation activity was still higher than that of *P. schreberi*, probably because water balance of non-branching acrocarps depend primarily on shoot density (Niinemets and Tobias, 2019). Species with an extremely high frequency of small leaves have shoots with large surface area, which increase water absorption (Larson, 1981), and provide more potential colonization sites for cyanobacteria than those mosses with fewer, larger leaves.

The cyanobacterial infective units, hormogonia, are of higher tolerance than other cyanobacterial cells and could be less affected by phenols while colonizing (Damerval *et al.*, 1991), but once differentiated to N$_2$-fixing heterocytes, phenols can inhibit N$_2$ fixation activity. However, a study of cycad–cyanobacteria symbiosis suggested that phenols provide a mechanism for excluding other microbes and permitting cyanobacteria to grow in cycad roots (Obukowicz *et al.*, 1981). Further study is needed to disentangle the effects of phenolics and other secondary compounds on moss and cyanobacteria associations. The studied mosses, with pH ranges from 5.0 to 6.0, might limit the activity of cyanobacteria, since the optimum pH for N$_2$ fixation in mosses is between 5.9 and 6.2 (Smith, 1984). However, neither N$_2$ fixation nor colonization was significantly affected by pH in our study. The pH of the moss with the highest cyanobacterial colonization and activity, *H. splendens*, ranged between 5.28 and 5.72, which is similar to the pH of the least colonized moss, *P. schreberi*, whose pH ranged between 5.32 and 5.64. This suggests that pH is not the most important determinant of cyanobacterial colonization and activity given the moss pH does not drop below 5.

### The role of ecosystem types

We note that a potential weakness of our trait analyses is that they included both inter- and intraspecific variation. We argue that trait correlations were mainly driven by interspecific variations. Samples of each species were harvested in the same habitat, and intraspecific trait variations, which are attributed to environmental factors should be small (Roos *et al.*, 2019). The analyses of CVs

**Phenol content and pH effects on cyanobacterial colonization**

In our experiment, phenols affected cyanobacterial activity negatively, but not cyanobacterial colonization. This fits with previous findings showing that moss phenol content did not correlate with cyanobacterial colonization (Rousk *et al.*, 2013a).
Fig. 8 Differences in acetylene reduction rate (AR, nmol g DW−1 h−1), colony structure (F_{sh}, frequency of shoots and H_{colony}, colony height), pH, phenol content, nitrogen content ([N]) and water balance-related traits (WC_{max}, maximum water content, W_{absorb}, time for 50% hydration and W_{lose}, time for 50% desiccation) of *H. splendens* between the two sampled sites (arctic tundra and boreal forest).

Fig. 9 Cyanobacteria colonization (%) on leaves and paraphyllia of *H. splendens*. The left figure compares the frequency of colonized leaves with the frequency of colonized paraphyllia. Each coloured dot represents a respective section from one *H. splendens* shoot; bars show the mean ± s.e. (n = 9). Frequency of cyanobacterial colonization was significantly higher on leaves than on paraphyllium (P = 0.003, two-way ANOVA). The picture on the right shows cyanobacteria (bright orange dots) on *H. splendens* leaves and paraphyllia, and the picture in the top-right corner shows cyanobacteria attached to a paraphyllium. Photos were taken under an Olympus BX61.
of traits confirmed that interspecific variations of traits were generally smaller than interspecific variations (Supplementary Data Table S1). For instance, the intraspecific CV of *P. schreberi* leaf frequency is significantly smaller than interspecific CV (0.12 versus 0.82, *P* = 0.04, modified signed-likelihood ratio test). Moreover, to demonstrate the intraspecific variation of traits and N₂ fixation rate, we compared several traits of *H. splendens* between ecosystem types (subarctic tundra versus boreal forest). *Hylocomium splendens* colonies from the tundra were shorter and had more shoots per area than the colonies from the forest (Fig. 7). These differences between ecosystems are likely driven by environmental factors, such as temperature, moisture (Bisbee *et al.*, 2001) and nutrient availability, as the tundra has a lower mean annual temperature (0.2 versus 1 °C) and precipitation (337 versus 570 mm) than the forest site (Rousk *et al.*, 2014; Rousk and Michelsen, 2017), impacting moss growth. Shoot size controls water fluxes in moss colonies (Elumeeva *et al.*, 2011) (see section: Linking hydration rate and cyanobacterial colonization), and can thereby control cyanobacterial colonization. Fine-scale variations in colony structure, such as changes in shoot frequency, alter surface roughness and further interact with wind flow affecting boundary-layer properties and desiccation rate (Rice and Schneider, 2004; Michel *et al.*, 2012). This is confirmed by the distinct desiccation rate for *H. splendens* colony between tundra and forest sites (Fig. 7). It is possible that trait variation, driven by ecosystem specific factors (e.g. N availability, temperature), are responsible for intraspecific differences in cyanobacterial colonization and thus N₂ fixation, while relative interspecific variation should remain similar in ecosystems with different abiotic conditions.

We did not assess cyanobacterial colonization for *H. splendens* from the forest site, but given that N₂ fixation activity is closely linked to cyanobacterial colonization, and activity was not different between the sites, it is likely that colonization is similar between the sites, too (Fig. 8). Moreover, in contrast with *H. splendens* collected from the forest site, *P. schreberi* had still lower N₂ fixation activity (27.75 versus 0.68 nmol g⁻¹ DW h⁻¹, Fig. 3). Hence, the variation in cyanobacterial colonization and, thus N₂ fixation activity, should be largely controlled by species-specific traits, but intraspecific variation that affects colonization is also in turn driven by the environment.

**Morphological peculiarity of *H. splendens***

Our results present the first evidence that cyanobacterial colonization of paraphyllia can be substantial. About 20–30 % of paraphyllia were colonized by cyanobacteria (Fig. 9). Given the large number of paraphyllia along the moss shoot, associated cyanobacteria can account for a considerable portion of N₂ fixation. Hence, focusing on moss leaves as colonization sites could vastly underestimate cyanobacterial colonization along moss shoots. Paraphyllia occur in taxa of only a few moss families, such as *Hylocomiaceae*, *Thuidiaceae* and *Brachytheciaceae* (Spirina *et al.*, 2020). If those taxa that have paraphyllia host more cyanobacteria than taxa without these structures is an open question. Moreover, paraphyllia, which are photosynthetic filaments or leaf-like structures composed of live cells, are located between leaf and stem, where many N₂-fixing cyanobacteria occur. Although these thick-walled cells may have low nutrient exchange rates, it is possible that paraphyllia could promote N uptake by the moss host by creating a link between epiphytic cyanobacteria and the moss stem. These open questions and unknowns call for further research on the paraphyllium’s role in the mosses’ N uptake, which could act as a lens through which to resolve the link between traits and ecological functions.

**In conclusion,** our findings emphasize that the hydration rate of a moss colony is a key trait regulating cyanobacterial colonization. On the other hand, species-specific morphological traits control hydration rate. Chemical traits of the moss host seem to be less important than morphological traits in regulating cyanobacterial colonization. We could also demonstrate that a considerable portion of cyanobacteria colonize paraphyllia, a previously overlooked structure in terms of cyanobacterial colonization. Variation in moss species-specific traits drives cyanobacterial colonization, but intraspecific variation that affects colonization is in turn driven by environmental factors. Given that mosses are a key source of N to ecosystems where they dominate the ground cover, uncovering the relationship between moss traits and cyanobacterial colonization will ultimately result in a better estimation of the amount of N input – as dependent on moss traits – and can provide new perspectives and information for understanding the relationship between mosses and cyanobacteria.

**SUPPLEMENTARY DATA**

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: pictures of the studied moss species. Figure S2: change in moss colony weight over time for four moss species during water absorption and loss. Figure S3: acetylene reduction rate in relation to the cyanobacteria count and density on moss leaves. Figure S4: relationship between leaf area and frequency of leaves for individual moss shoots. Figure S5: relationships between cyanobacterial colonization and shoot traits. Figure S6: differences in frequency of colonized leaves between top segments and lower segments. Figure S7: relationships between cyanobacterial colonization and leaf size. Table S1: intra- and interspecific CVs of water balance, colony, chemical and morphological traits.

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declare that they have no conflicts of interest with the contents of this article.

LITERATURE CITED


