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ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

The relationship of C and N stable isotopes to high-latitude moss-associated N_2 fixation

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Abstract

Moss-associated N_2 fixation by epiphytic microbes is a key biogeochemical process in nutrient-limited high-latitude ecosystems. Abiotic drivers, such as temperature and moisture, and the identity of host mosses are critical sources of variation in N_2 fixation rates. An understanding of the potential interaction between these factors is essential for predicting N inputs as moss communities change with the climate. To further understand the drivers and results of N_2 fixation rate variation, we obtained natural abundance values of C and N isotopes and an associated rate of N_2 fixation with $^{15}N_2$ gas incubations in 34 moss species collected in three regions across Alaska, USA. We hypothesized that $\delta^{15}N$ values would increase toward 0‰ with higher N_2 fixation to reflect the increasing contribution of fixed N_2 in moss biomass. Second, we hypothesized that $\delta^{13}C$ and N_2 fixation would be positively related, as enriched $\delta^{13}C$ signatures reflect abiotic conditions favorable to N_2 fixation. We expected that the magnitude of these relationships would vary among types of host mosses, reflecting differences in anatomy and habitat. We found little support for our first hypothesis, with only a modest positive relationship between N_2 fixation rates and $\delta^{15}N$ in a structural equation model. We found a significant positive relationship between $\delta^{13}C$ and N_2 fixation only in Hypnales, where the probability of N_2 fixation activity reached 95% when $\delta^{13}C$ values exceeded − 30.4‰. We conclude that moisture and temperature interact strongly with host moss identity in determining the extent to which abiotic conditions impact associated N_2 fixation rates.

Keywords Nitrogen · Bryophytes · Arctic · Boreal · Alaska

Introduction

Moss-associated nitrogen (N_2) fixation is the largest source of N inputs to boreal and Arctic ecosystems (Alexander and Schell 1973; Basilier 1979; DeLuca et al. 2002; Lindo et al. 2013; Vile et al. 2014). High northern latitude ecosystems, which are currently experiencing rapid changes associated with climate warming, harbor large reservoirs of carbon (C) and are often N limited for vascular plants (Shaver and Jonasson 1999; Lebauer and Treseder 2008; Tarnocai et al. 2009; Hugelius et al. 2014). While the N_2 fixation rates associated with the autotrophic microbes in the bryosphere are relatively low compared to symbiotic N_2 fixers associated with angiosperms such as *Alnus* spp. or cyanolichens such as *Peltigera* spp., the ubiquity of mosses in these ecosystems compensates for lower process rates (Hobbie et al. 2005; Weiss et al. 2005; Mitchell and Ruess 2009; Turetsky et al. 2010). Many biotic and abiotic variables have been proven to affect rates of moss-associated N_2 fixation directly and

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indirectly, including temperature, moisture, and N deposition (Ackermann et al. 2012; Gundale et al. 2012, 2013; Lindo et al. 2013; Rousk et al. 2013; Kardol et al. 2016; Salemaa et al. 2019). Other research shows that host moss identity can drive microbial community composition, *nifH* gene expression, and associated rates of N_2 fixation (Ininbergs et al. 2011; Bragina et al. 2012; Warshan et al. 2016; Holland-Moritz et al. 2018; Jean et al. 2020; Stuart et al. 2020). Previous measurements made in three Alaska ecoregions found consistent and significant differences in associated N_2 fixation rates among moss genera (Stuart et al. 2020). As abiotic conditions as well as moss biomass and community composition are expected to change with climate, understanding the interaction between environment and host moss identity is critical for predicting future N inputs (Gundale et al. 2012; Turetsky et al. 2012; Deane-Coe et al. 2015; Carrell et al. 2019).

The natural abundance of ^{15}N in plant material can provide clues regarding the source of plant N (Högberg 1997). For example, nitrogenase, the enzyme responsible for biological N_2 fixation, has a low discrimination against ^{15}N, leading to a $\delta^{15}N$ near − 1‰ or 0‰ for plants that are utilizing fixed N (Vitousek et al. 1989; Högberg 1997). In contrast, other plant N sources from soils or atmospheric N deposition are relatively enriched or depleted, respectively (Nadelhoffer et al. 1996; Bragazza et al. 2005). In mosses, natural abundances of $\delta^{15}N$ vary across ecological gradients, experimental treatments, and moss species (Bragazza et al. 2005; Gavazov et al. 2010; Deane-Coe et al. 2015). Critically, mosses obtain N fixed by microbial epiphytes (Bay et al. 2013; Berg et al. 2013; Rousk et al. 2016). Previous studies have used moss $\delta^{15}N$ as a potential reflection of differences in associated N_2 fixation rates across space or potential hosts by assuming signatures increasing toward 0‰ from more depleted values ($\delta^{15}N < -3$‰) had a higher N contribution via fixation, usually in combination with moss N concentrations, atmospheric N deposition signatures, and/or observed cyanobacteria colonization (Boddey et al. 2000; Deane-Coe and Sparks 2016; Novak et al. 2016; Živković et al. 2017).

Few studies have explicitly linked quantifications of moss-associated N_2 fixation to $\delta^{15}N$ signatures of the host mosses. In *Sphagnum* spp., more depleted (0 to − 3‰) $\delta^{15}N$ measurements were associated with higher rates of N_2 fixation (Leppänen et al. 2015), but in *Pleurozium schreberi* no relationship between these measurements was found (Hyodo et al. 2013). This discrepancy may be because the key measurements were made asynchronously or use only one natural abundance value linked with multiple N_2 fixation measurements. Mosses are also morphologically and ecologically hetereogeneous, and may vary in features that lead to greater soil N uptake or the translocation of N within the moss which can obscure recent inputs from N_2 fixation or other sources (Eckstein and Karlsson 1999; Aldous 2002; Bragazza et al. 2005; Ayres et al. 2006; Krab et al. 2008). Additionally, alternative forms of nitrogenase, such as vanadium (V)- and iron (Fe)-only nitrogenase, differ from molybdenum (Mo) nitrogenase in their levels of fractionation and thus their natural abundance signature, where V- and Fe-nitrogenase produce $\delta^{15}N$ signatures of − 6 to − 7‰ (Zhang et al. 2014). Sampled lichen thalli from Alaska fell mostly, though not exclusively, above the Mo concentration threshold which would indicate an increased reliance on V-nitrogenase activity (Darnajoux et al. 2019). Experimental Mo additions have shown a brief positive effect on N_2 fixation in subarctic feather mosses, but overall Mo concentration in control plots also fell above the threshold suggested by cyanolichen research (Rousk and Rousk 2020). By directly pairing N_2 fixation and natural abundance measurements across a broad diversity of host mosses and environmental conditions, we can more effectively explore the link between process rates and $\delta^{15}N$.

Given the key role that climate has in affecting N_2 fixation, another tool for exploring N_2 fixation rate variation is $\delta^{13}C$. In mosses, $\delta^{13}C$ reflects temperature, moisture conditions, and plant productivity, both in natural and experimentally induced conditions (Williams and Flanagan 1996; Skrzypek et al. 2007; Deane-Coe et al. 2015; Royles et al. 2016; Granath et al. 2018). Unlike vascular plants, where fractionation against ^{13}C isotope occurs both in diffusion through stomata and enzymatic discrimination, mosses in moist environments often have a film of water on the surface which limits diffusion (Farquhar et al. 1989; Williams and Flanagan 1996; McCarroll and Loader 2004). Thus, while $\delta^{13}C$ in vascular plants becomes relatively depleted with increased temperature/decreased precipitation, mosses are relatively more enriched in ^{13}C in moist conditions, as limited diffusion decreases enzymatic discrimination (Stuiver and Braziunas 1987; Williams and Flanagan 1996; Rice 2000; Diefendorf et al. 2010). Therefore, $\delta^{13}C$, which is often obtained concurrently with $\delta^{15}N$ analysis, is linked to climatic drivers of N_2 fixation.

The objective of our study was to evaluate the relationships between $\delta^{13}C$, $\delta^{15}N$, and N_2 fixation in high-latitude mosses to identify links between stable isotope composition and process rates as well as to explore the interaction of host moss identity with these relationships. We used $^{15}N_2$ incubations to measure the fixation rates associated with 34 different moss species across a latitudinal transect in Alaska from 60° to 68° N, each with a paired natural abundance measurement from the same sample pre-incubation. We hypothesized that moss $\delta^{15}N$ would increase toward 0‰ with increasing N_2 fixation (H1). Past studies have shown a positive relationship between cyanobacteria colonization and $\delta^{15}N$ and that mosses utilize N fixed by microbial epiphytes (Berg et al. 2013; Deane-Coe and

Sparks 2016). We also hypothesized that more enriched δ^{13}C would have a positive relationship with rates of N_2 fixation (H2), as higher δ^{13}C can indicate consistent moist conditions that facilitate microbial N_2 fixation. Finally, we expected that these relationships would vary in magnitude among mosses. In addition to known differences in anatomy and habitat niche differentiation related to nutrient acquisition and water retention strategies, previous analysis of these samples indicated the key role of host identity in determining associated rates of N_2 fixation (Stuart et al. 2020). To test these hypotheses, we used mixed models and a multi-group structural equation model after first using exploratory random forest models to inform tested relationships. We also used a binomial generalized mixed-effects model to establish a broad threshold of for N_2 fixation activity related to δ^{13}C signatures.

Materials and methods

Sites

In June 2016, we selected ten sites near Fairbanks, AK (N 64° W 148°) and four sites near Toolik Field Station, AK (N 68° W 149°). The following June, we selected an additional ten sites near Anchorage, AK (N 60–61°, W 149°). These sites encompassed a diversity of ecotypes found in the regions, including *Picea* spp. dominated upland and peatland boreal forest, alpine tundra, *Tsuga* spp. coastal forests, and Arctic tundra. Sites were selected based on accessibility, low human disturbance, and the presence of moss. Each site was based on a 30 m transect where environmental measurements were made at 5 m intervals, hereafter called subplots. For more details on the location and characteristics of sites, please see Stuart et al. (2020).

Environmental data collection

Gravimetric water content of the moss was measured via a 5 × 5 × 5 cm plug of moss from each subplot. These plugs were placed in an airtight plastic bag to be transported to the laboratory and immediately weighed for a field wet weight before being transferred to a 60° drying oven for 48 h before reweighing for a dry weight. Moisture, or gravimetric water content, was calculated by dividing the difference between the wet and dry weights by the dry weight. Coordinates and elevation were recorded for each site using a Garmin GPSMAP 64 × handheld GPS. Coordinate information was then used to extract the mean annual temperature (MAT, in °C) and precipitation (MAP, in mm) for each site from 2014 to 2017 from Climate NA (Wang et al. 2016).

Relative abundance of Nostocaceae

To compare our results to papers that looked only at cyanobacteria colonization, we compared Nostocaceae relative abundance with N_2 fixation rates and δ^{15}N in linear models. For full details of microbial methods, please see Holland-Moritz et al. (2021). Briefly, we used amplicon-based sequencing of a 253 bp region of the 16S rRNA bacterial and archaeal marker gene on a third subsample paired with the natural abundance and N_2 fixation measurement samples described above. We extracted DNA from moss tissue, homogenized with liquid N_2 and PCR-amplified the V4-V5 region of the 16S rRNA gene. Amplicons were sequenced on the Illumina MiSeq platform at the University of Colorado Next Generation Sequencing Facility using 2 × 150 bp paired-end chemistry. After sequencing we identified microbial phylotypes using the UNOISE pipeline (Edgar 2018) which denoises the reads and distinguishes between phylotypes using a unique sequence variant approach (i.e. all sequences belonging to a single phylotype have 100% identical sequences). We assigned taxonomy to these phylotypes with the RDP Naive Bayesian Classifier (Wang et al. 2007) and GreenGenes database (McDonald et al. 2012). After filtering out phylotypes assigned as mitochondrial or chloroplast, we created a phylotype-by-sample table and controlled for differences in sequencing depth across samples by randomly selecting 3000 reads per sample. Finally, we converted our read-counts to relative abundances and calculated the percent relative abundance of Nostocaceae in each sample.

N_2 fixation and natural abundance measurements

All mosses at each site were identified and, if they appeared in at least two distinct patches, collected in each subplot where present. Common mosses, appearing in at least six distinct patches, were collected a maximum of six times (once per subplot). For each sample, circa 40 moss ramets were collected. From this original sample, several stems were removed for a voucher specimen to be identified at the University of Florida, ten stems were immediately placed in a drying oven for natural abundance values, and ten stems were placed in syringes to be incubated with $^{15}N_2$ gas. Each moss ramet included was of approximately 5 cm of length, including both green and senescent moss tissue, and was cleaned of debris or other plant material. Natural abundance samples were dried at 60 °C for 48 h before being shipped to Northern Arizona University. The incubation subsample was lightly sprayed with distilled water in an airtight 60 ml polypropylene syringe. Each syringe was plunged to 10 ml of ambient air before 10 ml of 98 at% enriched $^{15}N_2$ gas was added, making the final volume 20 ml (Sigma-Aldrich Inc., Lot # MBBB3807V and MBBB9003V). Incubations took place in a common garden (one in each geographic area of Toolik, Fairbanks, and Anchorage) for 24 h, as past research showed no significant difference between in situ and common garden incubations (DeLuca et al. 2007). Three additional syringes, each containing a Thermochron iButton (Model DS1921G-F5#, Embedded Data Systems USA), recorded field temperature every 10 min throughout the duration of the incubation. After incubation, moss material was removed from the syringes, dried as described above, and sent to Northern Arizona University.

Natural abundance and incubated samples were finely ground using either a clean coffee grinder or hand-chopped with scissors. Six mg of each sample was rolled into a tin capsule and run with a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer to obtain %N, %C, $\delta^{15}N$, and $\delta^{13}C$. The atom percent enrichment (APE) of each sample was calculated by subtracting the natural abundance subsample atom percent from its paired incubated subsample atom percent. Isotopic uptake was then scaled by the sample weight and the air:tracer ratio to calculate total ($^{15}N + ^{14}N$) N_2 fixation (Jean et al. 2018). Rates are expressed as μg N g moss^{-1} day^{-1}.

We measured both natural abundance values and N_2 fixation rates associated with 505 samples. The average natural abundance and enriched $\delta^{15}N$ was $-3.09 \pm 0.08‰$ (mean ± standard error) and $54.67 \pm 4.05‰$, respectively. Natural abundance of $\delta^{13}C$ averaged -30.81 ± 0.10 and percent N and percent C were 0.81 ± 0.01 and 46.45 ± 0.11, respectively. Based on the sensitivity of the isotope ratio mass spectrometer, samples with $< 2‰$ $\delta^{15}N$ difference between the paired samples were assumed to have a N_2 fixation rate of zero. For more detail on the past analyses of these samples, please see Stuart et al. (2020). Note that results presented in this manuscript include only samples that had both a measurement of N_2 fixation and a directly paired natural abundance value. In the previous study, some samples without a paired natural abundance value were included based on a site-level, moss species-specific average natural abundance value.

Statistical analyses

All statistical analyses were performed in R Studio 1.2.1335 using R version 3.6.1 (R Core Team 2017). To test the hypotheses of this study, we used piecewise structural equation models (SEMs) through the package piecewiseSEM 2.1.0 (Lefcheck 2016) and linear mixed-effects models in lme4 1.1-21 (Bates et al. 2015) paired with Satterthwaite's degrees of freedom method in lmerTest (Kuznetsova et al. 2017). Prior to implementing SEMs, we used random forests in the package randomForest 4.6–14 to explore both linear and non-linear relationships for each of the three response variables we were interested in modelling as endogenous variables within the SEM framework: $\delta^{13}C$, $\delta^{15}N$, and N_2 fixation rate (Liaw and Wiener 2002). Random forests are a flexible and informative approach in variable selection as they can incorporate both continuous and categorial variables and do not rely on assumptions of frequentist statistics (Cutler et al. 2007). As previous results demonstrated the importance of host moss identity in determining rates of N_2 fixation, we divided mosses into genera or orders for analyses: Hypnales (containing the genera *Hylocomium*, *Ptilium*, *Pleurozium*, *Hypnum*, *Tomentypnum*, *Rhytidiadelphus*, and *Sanionia*), Sphagnales (including 11 different species of *Sphagnum*), Rhizogoniales (three *Aulacomnium* spp.), Dicranales (eight *Dicranum* spp.), and Polytrichales (three *Polytrichum* spp.). Therefore, the only order than contains multiple genera is Hypnales. We grouped our samples to gain statistical power and we use the ordinal names here as a convenience. However, we note that our conclusions are specific to these samples and do not necessarily characterize these physiological processes in the orders as a whole. Past analysis suggests that lower taxonomic levels are likely to possess ecologically relevant rate variation in N_2 fixation (Stuart et al. 2020). We compared the R^2 values between two different models for each endogenous variable, where one model contained moss genus as a predictor and the other substituted order for genus, to determine whether it was appropriate to test our hypotheses at a broader taxonomic scale. Each model also contained MAP, MAT, altitude, incubation temperature average, gravimetric water content, site, geographic area (Toolik, Fairbanks, or Anchorage), and the other endogenous variables as predictors. Each model averaged six executed runs with mtree set at 10,000.

After confirming the limited reduction of explained variance when using host moss order instead of genus, we implemented two mixed models. To account for spatial autocorrelation, we included the geographic area (Anchorage, Toolik, or Fairbanks), site nested in geographic area (24 sites, described above) and subplot nested in site and geographic area (six subplots per site) as random effects. Interactive fixed effects of $\delta^{13}C$ and order were regressed with the natural log of (N_2 fixation rate (μg N g moss^{-1} day^{-1}) + 1), while interactive fixed effects of N_2 fixation rate and order were used with $\delta^{15}N$ as the response variable. $\delta^{13}C$ was treated as the independent variable because it is a reflection of climate conditions, which in turn is hypothesized to relate to rates of N_2 fixation. Conversely, N_2 fixation rate was the independent variable when regressed with $\delta^{15}N$ because the natural abundance of N isotopes may reflect rates. Model results are shown with transformed and back-transformed predicted relationships implemented through the ggeffects package 1.0.2 (Lüdecke 2018). For the Hypnales, we also ran a generalized linear mixed-effects model on a binomial dataset, where N_2 fixation rates were allocated to zero if no fixation was observed and one if fixation did occur during the incubation. We were unable to test the effect of geographic area because all samples from Toolik fixed N_2 during our incubation (see Supplementary Fig. 1). We regressed the presence/absence of N_2 fixation activity against the $\delta^{13}C$ natural abundance value in a binomial model with a bobyqa optimizer with random effects of site and subplot nested in site.

We used SEMs to further test our initial hypotheses for Hypnales and Sphagnales. The a priori model included MAT and MAP as exogenous predictors of $\delta^{13}C$ and ln (N_2 fixation rate + 1) and %N as an exogenous predictor of $\delta^{15}N$ and ln (N_2 fixation rate + 1) (see Fig. 2). We opted for MAT and MAP indicators over snapshot measurements of temperature and moisture made at the time of sampling as they may correspond better with $\delta^{13}C$ values that reflect longer term trends. Additionally, we previously found that MAT and MAP were generally better predictors of N_2 fixation rates than the snapshot measurements (Stuart et al. 2020). Measures that combine temperature and precipitation, such as climate moisture deficit, were considered but ultimately rejected to enable looking at each driver separately, as moisture has a consistently positive effect on N_2 fixation while temperature optima can differ among N_2 fixers (Gentili et al. 2005; Rousk et al. 2013). While %N could be a result of, rather than a cause of, N_2 fixation rate variation, we worked under the assumption that increased N deposition would act more directly on %N and thus downregulate fixation activity (Solga and Fram 2006; Gundale et al. 2013; Salemaa et al. 2019). This explanation seems plausible in our data, given the weakly negative relationship between %N and N_2 fixation in our data. In the SEM, N_2 fixation rates + 1 were natural log-transformed to meet model assumptions. A multi-group SEM was employed to directly compare paths between Hypnales and Sphagnales within the same model. A multi-group piecewise SEM allows a direct comparison between two groups within the same model by allowing paths to vary if there is a significant interaction between the term and the grouping factor. The package automatically tests which paths to constrain, with selections made for the best output. The piecewise SEM also allows for the inclusion of the hierarchical sampling design, as each relationship includes the random effect structure described for mixed-effect models above.

Results

Across all three variables of interest ($\delta^{13}C$, $\delta^{15}N$, and N_2 fixation), the substitution of moss order for moss genus as a predictor resulted in small or no change to the amount of variance explained by the model (Table 1). The largest diminution of explained variance between two models for the same variable of interest was for N_2 fixation rate, where the model including genus explained 9% more variation. Across all models, moss identity (genus or order) was the top-ranked predictor. Similarly, all models showed some influence of site of collection and relatively little influence from the geographic area (Toolik, Fairbanks, or Anchorage).

Table 1 Comparison of random forest R^2 for modeling variables of interest with different moss taxonomic levels as a predictor with the same suite of environmental predictors (including MAT, MAP, altitude, incubation temperature average, gravimetric moss water content, site, and geographic region)

Predicted variable	Model R^2	
	Moss genus	Moss order
$\delta^{13}C$ (‰)	0.651	0.650
N_2 fixation rate (μg N g moss^{-1} day^{-1})	0.422	0.333
$\delta^{15}N$ (‰)	0.551	0.561

Mixed model analysis showed a significant interaction between $\delta^{13}C$ and moss order ($p < 0.001$, Fig. 1, Table 2). Orders Hypnales and Sphagnales were significantly different from the intercept (95% Confidence Interval (CI) = 0.07–0.27, $p = 0.006$ and CI = − 0.28 to − 0.03, $p = 0.013$, respectively). Post hoc graphing of model results showed that the direction of the relationship between $\delta^{13}C$ and transformed N_2 fixation rate was different between these orders; while Hypnales displayed the expected positive relationship, Sphagnales had a negative relationship (Fig. 1). Other moss orders did not have a significant relationship between $\delta^{13}C$ and transformed N_2 fixation rates, though the direction of those relationships was positive (Fig. 1). Random effects testing confirmed that geographic area and site nested in geographic area accounted for 32% and 14% of variation explained respectively, while subplot was not significant and accounted for < 3% of variation. The interaction between the transformed N_2 fixation rate and order was significant when regressed with $\delta^{15}N$, but only significant for Polytrichales (Table 2, Fig. 1). The $\delta^{15}N$ values of Polytrichales and Rhizogoniales were significantly higher than other moss orders. Only site nested in geographic area was significant as a random effect and accounted for 33% of variance explained.

Based on mixed model results, ecological prevalence, and higher associated N_2 fixation rates within Hypnales and Sphagnales, these two orders were compared in a multi-group SEM. All but four relationships were constrained to the global model (Fig. 2). Therefore, there was a significant interaction between moss order and $\delta^{13}C$ in relation to ln (N_2 fixation + 1), percent N in relation to $\delta^{15}N$, and MAT in relation to $\delta^{13}C$ and ln (N_2 fixation + 1). For Sphagnales, MAT was negatively related to $\delta^{13}C$ and, unlike the mixed model results, transformed N_2 fixation rate had a significant

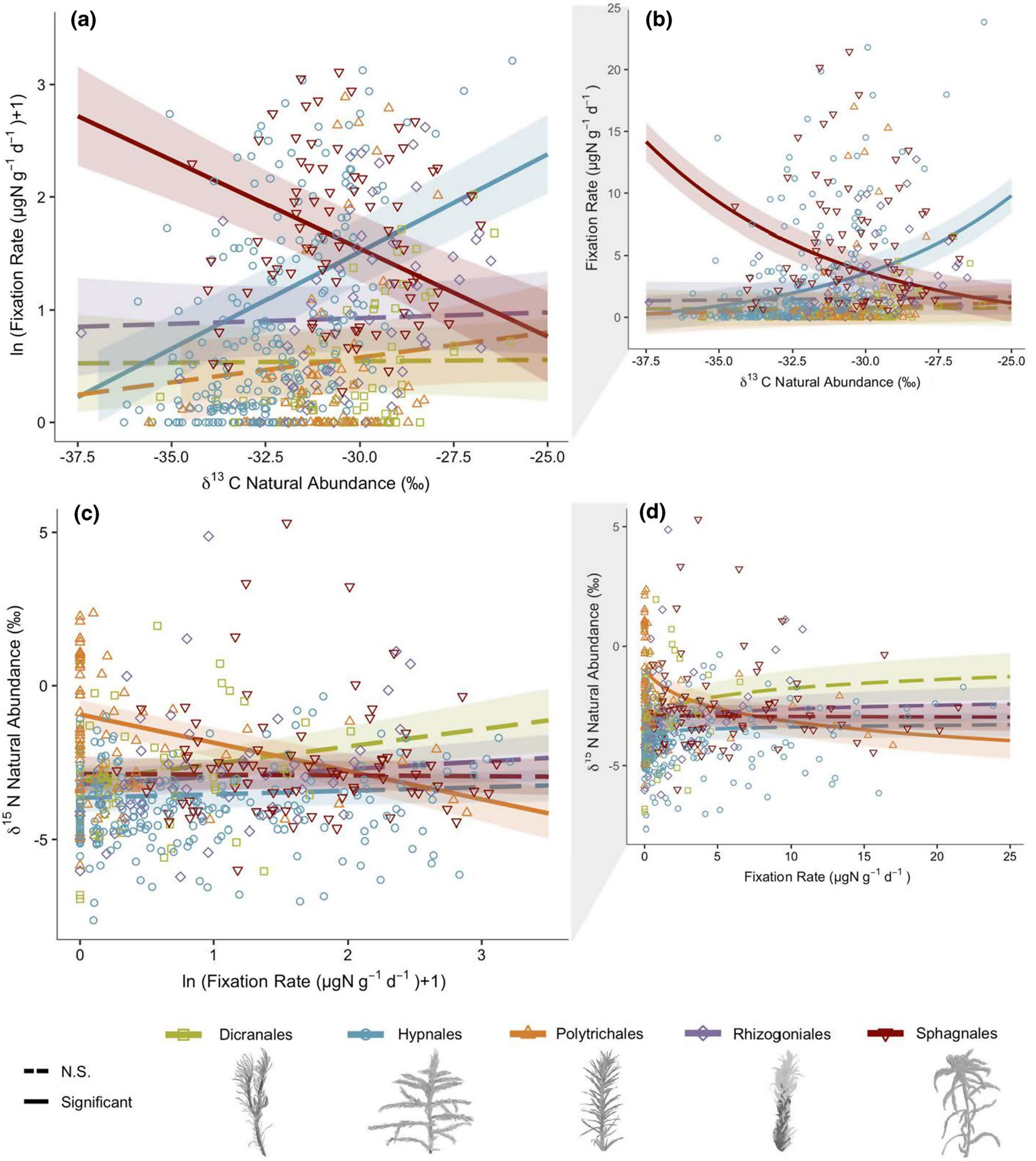

Fig. 1 Panel **a** shows estimated marginal means predictions of mixed model results regressing the interaction between moss order and $\delta^{13}C$ with transformed N_2 fixation rate (ln (μg N g moss^{-1} day^{-1} + 1). Panel **c** shows estimated marginal means predictions of mixed model results regressing the interaction between moss order and transformed N_2 fixation rate (ln (μg N g moss^{-1} day^{-1} +1) with $\delta^{15}N$. Lines represent predicted relationships, and ribbons are the 95% confidence interval of the predicted relationships. Both models included geographic region, site nested in geographic region, and subplot nested in site and geographic region as random effects. Panels **b** and **d** show estimated marginal means predictions for each model with ln (n+1) values back-transformed for graphing purposes for $\delta^{13}C$ and $\delta^{15}N$ model, respectively

Table 2 ANOVA tables (Type III with Satterthwaite method) for ln (N_2 fixation rate (μg N g moss^{-1} day^{-1}) + 1) as a function of the interaction of moss order and δ^{13}C (M1, see Hypothesis 2) and δ^{15}N as a function of the interaction of moss order and ln (N_2 fixation rate (μg N g moss^{-1} day^{-1}) + 1), (M2, see Hypothesis 1)

M1: ln (N_2 fixation + 1) model				M2: δ^{15}N model			
Model term	df	*F*	*p*	Model term	df	*F*	*p*
δ^{13}C	1	0.36	0.551	ln (N_2-fix + 1)	1	0.001	0.973
Order	4	10.48	**< 0.001**	Order	4	42.80	**< 0.001**
δ^{13}C:Order	4	10.55	**< 0.001**	Log_e (N_2-fix + 1):Order	4	6.98	**< 0.001**

Bold indicates *p*-values less than 0.05

Model marginal R^2 was 0.320 in M1 and 0.172 in M2

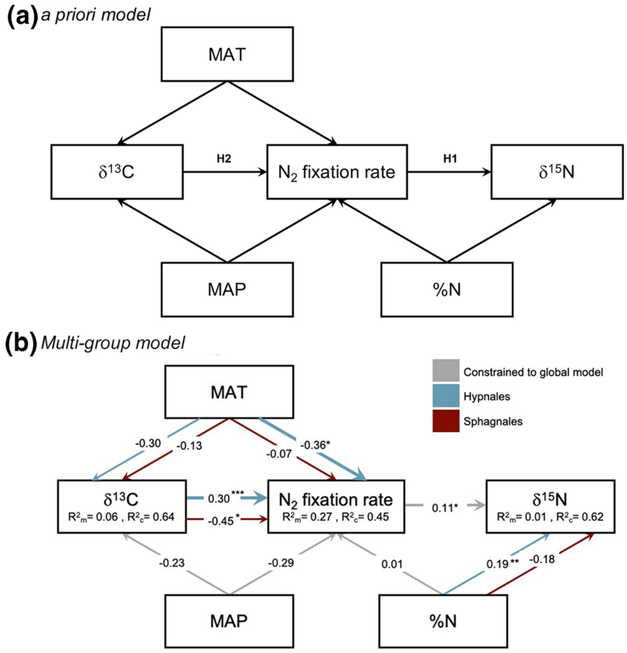

Fig. 2 Panel **a** is the a priori model of proposed relationships within the SEM, where the hypothesis-testing relationships are highlighted. Panel **b** shows the multi-group model results, including the marginal and conditional R^2 values of the endogenous variables. The numbers on each path are the path coefficients, with significant *p* values denoted with *($p < 0.05$) and ***($p < 0.001$). Model Fisher's C was 12.32 ($p = 0.138$, df = 8). MAT was measured in °C, MAP in mm, N_2 fixation rates in ln (μg N g moss^{-1} day^{-1} + 1), and δ^{13}C and δ^{15}N in ‰

relationship δ^{15}N ($P = 0.0156$). Other relationships, such as MAP and δ^{13}C or percent N and transformed N_2 fixation rate, were not significant in the model (Fig. 2).

The presence of N_2 fixation activity was modeled as a function of δ^{13}C values, which was found to be a significant relationship (CI = 1.12–2.12, $P = 0.008$, Fig. 3). Geographic area could not be included as a model term, because all moss samples from Toolik fixed N_2 during our incubations (see Supplementary Fig. 1). In the most depleted samples (− 36‰), mosses were slightly more likely to be fixing than not fixing N_2, though the confidence region crosses below the 50% threshold. However, at − 30.4‰, samples crossed the threshold of a 95% probability prediction. All samples in our study displayed N_2 fixation activity at δ^{13}C values more enriched than − 30‰. This trend did not appear to be driven by one moss species in particular, although *Tomentypnum nitens* had consistently high N_2 fixation rates and relatively depleted δ^{13}C (Fig. 3, Table 3).

Discussion

Our observations of a large paired dataset that compares moss-associated N_2 fixation rates with natural abundance stable isotope values revealed a surprising disconnect between δ^{15}N and rates of N_2 fixation. We found little evidence to support our hypothesis that δ^{15}N values would increase toward 0‰ with N_2 fixation activity, even after accounting for host moss identity. We found a significant interaction of δ^{15}N and transformed N_2 fixation rate which was due to the relationship in Polytrichales (Table 2, Fig. 1). Polytrichales had a generally higher δ^{15}N signature despite having consistently low N_2 fixation rates, resulting in a relationship of the opposite direction than we predicted in H1. Members of the Polytrichales have more developed internal transport systems, which perhaps facilitates greater soil uptake than other studied moss groups (Brodribb et al. 2020). The modest positive relationship between δ^{15}N and N_2 fixation in the SEM framework was significant for Hypnales and Sphagnales together. However, the actual δ^{15}N values were not close to − 1 or 0‰, instead remaining largely below − 2.25‰ (based on value of 3rd quantile when rate of fixation was greater than 5 μg N g moss^{-1} day^{-1}). This is in contrast to a previous study, which saw a positive connection between the percentage of leaves with cyanobacteria colonization and elevated δ^{15}N in temperate forest mosses (Deane-Coe and Sparks 2016). While relative abundance does not necessarily equate to absolute abundance, we did see a significant positive relationship between Nostocaceae relative abundance and transformed N_2 fixation rates but no relationship between Nostocaceae relative abundance

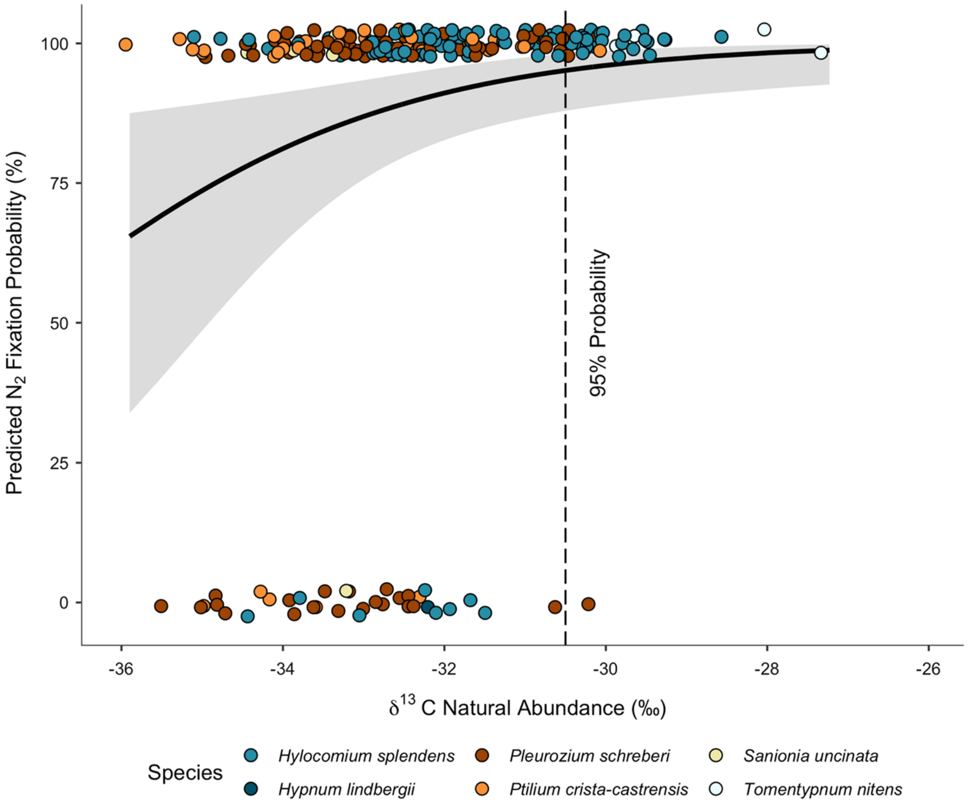

Fig. 3 N_2 fixation activity was modeled as a function of $\delta^{13}C$ with random effects of site and subplot nested in site, where rates of > 0 µg N g moss^{-1} day^{-1} were assigned a value of 1. Shading represents the 95% prediction intervals. Points are staggered for visual differentiation and colored based on moss species. Vertical dotted line represents the point on the x-axis at which the predicted probability reached 95%. Graph and model contained data from all three geographic areas included in the study (Anchorage, Fairbanks, and Toolik)

and $\delta^{15}N$ (Fig. 4). There is some evidence that N_2 fixation rates associated with bryophytes in temperate forests may be up to an order of magnitude greater than boreal or Arctic rates and have higher $\delta^{15}N$ signatures, perhaps reflecting the increased footprint of fixation (Menge and Hedin 2009; Menge et al. 2011). Lower rates of N_2 fixation relative to high latitudes have been measured in temperate grasslands in North America, and higher rates of N deposition in temperate ecosystems would be expected to lead to downregulation of the process (Gundale et al. 2013; Hember 2018; Calabria et al. 2020).

One explanation for the absence of our hypothesized relationship is the increased reliance on alternative nitrogenases at higher latitudes, which would be expected to produce a much different natural abundance signature (Zhang et al. 2014; Darnajoux et al. 2019). The use of $\delta^{15}N$ as a straightforward proxy is also complicated by other N acquisition strategies in mosses which may dilute the signature of fixed N, including atmospheric deposition, uptake from soil, and translocation of N from senescent to live tissue (Aldous 2002; Turetsky 2003; Ayres et al. 2006; Krab et al. 2008; Harmens et al. 2014). Rates of N deposition in Alaska are generally less than 2 kg N ha^{-1} year^{-1} and extractable inorganic N was generally low across sampled sites (Hember 2018; Stuart et al. 2020). In particular, throughfall N can be a large source of N for boreal mosses and may dilute the signal of N_2 fixation or contribute to process downregulation (Forsum et al. 2006; DeLuca et al. 2008). In experiments on *Sphagnum* spp. and *P. schreberi*, it seems likely that mosses obtain at least a portion of fixed N and that fixed N is retained in the green moss layer for at least 1 year, indicating the rapid loss of fixed N is an unlikely explanation for the weakness of the observed trend (Basilier 1980; Berg et al. 2013; Rousk et al. 2016). Based on our results, we cannot recommend using $\delta^{15}N$ as an indicator of N_2 fixation in high-latitude mosses without further elucidation of potential N sources and active nitrogenase forms.

Contrary to our initial prediction that host identity would impact only the magnitude of the hypothesized relationship between $\delta^{13}C$ and rates of N_2 fixation, we found instead that moss order was a key determinant of the presence and direction of significant relationships. A positive relationship between $\delta^{13}C$ and N_2 fixation was only significant in the feather moss order Hypnales. Previous research has confirmed the importance of moss identity in the composition of microbial communities and the associated rates of N_2 fixation (Ininbergs et al. 2011; Leppänen et al. 2015; Jean et al. 2020; Stuart et al. 2020; Holland-Moritz et al. 2021). Analysis of data from our sites found that moss taxonomic identity was the top-ranked predictor of associated N_2 fixation rates in all geographic areas (Stuart et al. 2020). However, site MAP and MAT were the third and fourth ranked predictors, respectively (Stuart et al. 2020). Our results here add evidence to the premise that the distinct microbial

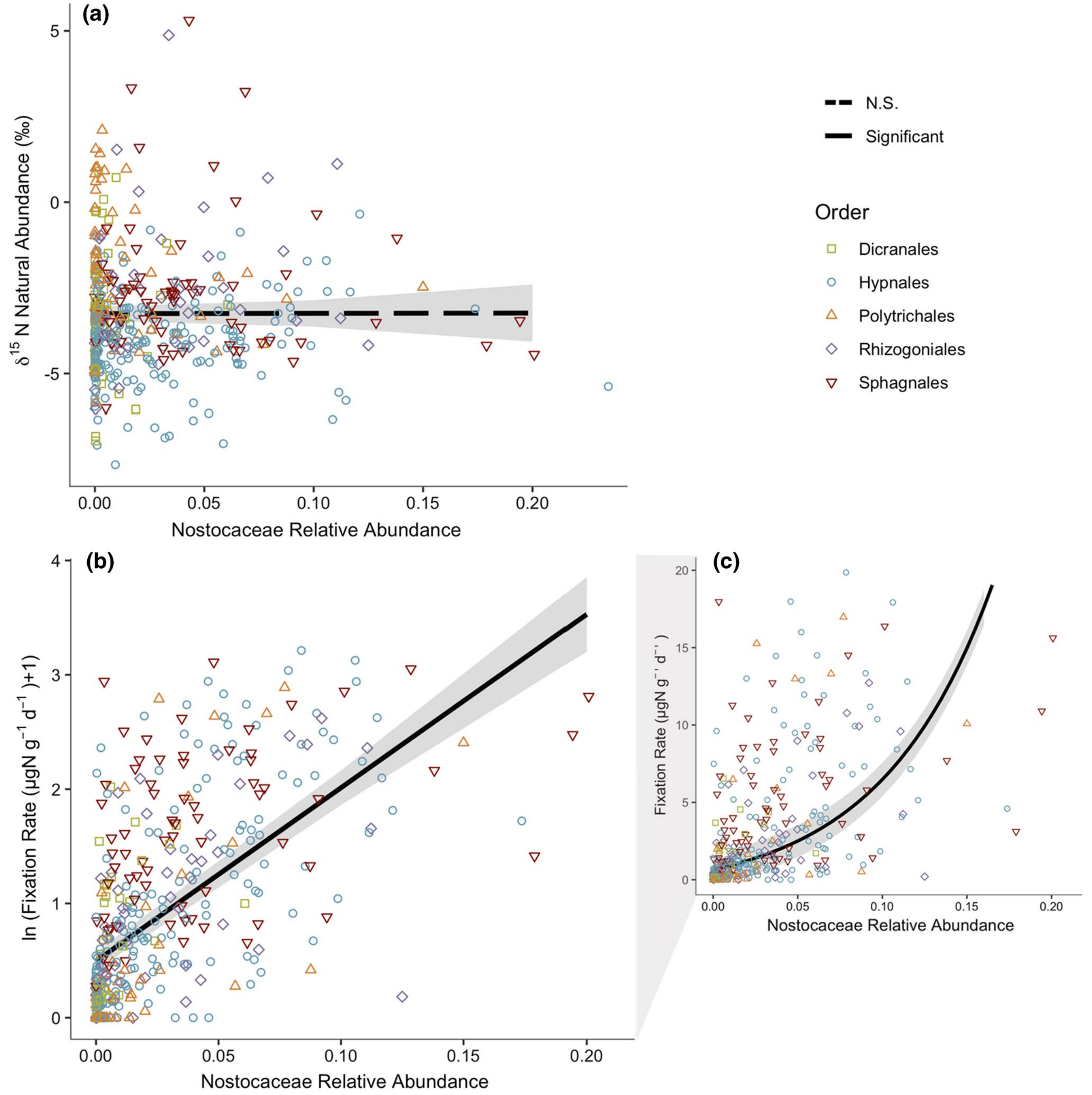

Fig. 4 Linear model results comparing the relative abundance of Nostocaceae in each sample with the corresponding δ^{15}N **a** or natural log of N_2 fixation rate (μg N g moss^{-1} day^{-1}+1) **b**. Panel **c** shows back-transformed values for the relationship between Nostocaceae relative abundance and N_2 fixation. Each point represents one sample. Solid lines represent a significant relationship ($p < 0.001$, $R^2 = 0.36$), and dotted line represents non-significant relationship ($p = 0.947$). Shaded area is 95% confidence interval. Colors represent taxonomic order, though order was not included as a model term

communities harbored by mosses will respond differently to key process drivers of moisture and temperature, and thus that the response of N_2 fixation to changes in climate will not be universal among mosses.

In agreement with previous studies, we found negative relationships between MAT and δ^{13}C for Hypnales (Skrzypek et al. 2007; Deane-Coe et al. 2015). However, the direction of this relationship was reversed in Sphagnales within our SEM (Fig. 2). The absence of the same relationship in Sphagnales may reflect the unique anatomy of these mosses which enables a high water-holding capacity (van Breemen 1995; Elumeeva et al. 2011). Additionally, the

Table 3 Mean N_2 fixation rate (μg N g $moss^{-1}$ day^{-1}) for each moss order across all sampling sites ± standard error with sample size (*n*)

Moss order	Mean N_2 fixation rate					
	Anchorage		Fairbanks		Toolik	
	μg N g $moss^{-1}$ day^{-1}	*n*	μg N g $moss^{-1}$ day^{-1}	*n*	μg N g $moss^{-1}$ day^{-1}	*n*
Dicranales	0.27 ± 0.14	21	0.40 ± 0.16	31	1.95 ± 0.35	20
Hypnales	1.74 ± 0.27	131	2.44 ± 0.43	78	9.41 ± 1.40	21
Polytrichales	0.12 ± 0.03	31	0.04 ± 0.02	19	5.01 ± 1.32	19
Rhizogoniales	2.08 ± 0.52	30	2.15 ± 0.77	13	3.71 ± 0.79	17
Sphagnales	5.27 ± 0.67	47	4.53 ± 0.85	22	9.31 ± 2.25	9

isotopic composition of the CO_2 available to mosses may be influenced by plant detrital inputs and microbial fractionation (Werth and Kuzyakov 2010). As Sphagnales create areas with low decomposition and high organic matter accumulation (Gorham 1991), this may play a role in the reversal of the expected relationship. The range of $\delta^{13}C$ values for Sphagnales was only − 34.0 to − 26.8‰, compared to − 37.4 to − 24.3‰ for all other measured mosses. Unlike all other moss orders, every collected *Sphagnum* moss had detectable N_2 fixation activity across its more constrained $\delta^{13}C$ range. The high moisture retention of Sphagnales may increase latent heat loss (Fukuta et al. 2012). Previous research has shown that *Sphagnum palustre* (in the order Sphagnales) can buffer air temperatures, particularly when air temperatures exceed 20 °C, while *Hylocomium splendens* (Hypnales) does not provide as much temperature insulation at the moss carpet (Sonesson et al. 1992; Fukuta et al. 2012). It follows that the trend between MAT and $\delta^{13}C$ would be more important in the Hypnales than the Sphagnales, which is borne out in our model. MAP was not significantly related to $\delta^{13}C$, but this may be due to sites which were in relatively hygric to mesic landscape positions. Only one site fell below the moisture threshold described by Zielke et al. (2005) as inhibiting N_2 fixation. For Hypnales and Sphagnales, $\delta^{13}C$ was a more significant predictor of N_2 fixation activity than either of the climate variables in our model, which may better reflect the synthesis of conditions within the moss carpet than temperature or precipitation averages.

Using a binomial distribution of N_2 fixation activity associated with Hypnales, we identified a threshold of $\delta^{13}C$ values at which our model predicted a 95% probability of N_2 fixation. Across the spectrum of $\delta^{13}C$, feather mosses were fixing N_2 during our incubations. However, in samples more enriched than − 30‰, all collected samples were fixing N_2 (Fig. 3). Due to the uniform presence of N_2 fixation activity at sites near Toolik, we were unable to account for the effect of geographic area in the binomial model. However, Toolik has a lower mean annual temperature than the other two sampling areas, and we observed generally more enriched $\delta^{13}C$ and higher N_2 fixation rates (Supplementary Fig. 1). Higher temperatures and/or lower moisture did not universally inhibit fixation activity; even at the most depleted $\delta^{13}C$ values, our model predicted a 62% probability of N_2 fixation activity. From our data, we infer that while relatively lower temperatures and higher moisture promote N_2 fixing activity, the opposite cannot be assumed.

One caveat to the research presented here is that each site was measured only once, providing a snapshot of N_2 fixation values. It is possible that the measured N_2 fixation rate at any given site was anomalous for the location or the host species in question, or that the present conditions that facilitated or diminished N_2 fixation activity did not correspond with the historical trend that would be encapsulated by the C and N stable isotope values we measured. The 5 cm section of moss measured in our sampling could represent 1–4 years of growth, depending on the moss species (Pakarinen and Rinne 1979), and inter- and intra-annual variation in N_2 fixation rates has been observed previously (Rousk and Michelsen 2017; Jean et al. 2018). By sampling from many sites across a broad scale of geographic locations, however, we present a large dataset which can partially account for natural variation. Our nested random effects structure explained a notable proportion of model variance. Comparing the marginal and conditional R^2 in the SEM also shows that the random effects were a considerable source of variation, particularly in the stable isotope signatures (Fig. 2). This suggests that the stable isotope signature had a stronger geographic bias than N_2 fixation rates, possibly due to the more integrative stable isotope measurement of weather conditions or N sources over time.

Though we assumed that the strength of hypothesized relationships of N_2 fixation and stable isotope signature would vary between different groups of host mosses, the absence of significant relationships and/or different directions of relationships among orders indicates the degree to which the anatomy, life history, or micro-environment of these mosses could influence our attempts to understand the drivers of N_2 fixation rate variation. The most striking example of this is the difference between the relationship of $\delta^{13}C$ to fixation activity in Sphagnales and Hypnales. Though moisture and temperature are generally considered to be primary drivers of N_2 fixation rate variation, only in

Hypnales did the expected positive relationship between a metric of moisture/temperature status and activity occur. While no significant trends were seen among the other moss orders included in this study, Dicranales, Polytrichales, and Rhizogoniales also had generally lower rates of N_2 fixation, making the trend harder to observe.

These observations underline the importance of thinking of moss identity as an interaction term along with environmental variables and, where possible, testing hypotheses with a diversity of host mosses. Both rates of N_2 fixation and moss community composition are expected to change with the climate (Turetsky et al. 2010; Gundale et al. 2012; Deane-Coe et al. 2015; Carrell et al. 2019). Moss-associated N_2 fixation, along with other biogeochemically relevant moss traits, plays an important role in high-latitude C balance (Cornelissen et al. 2007; Lindo et al. 2013). Interspecific knowledge and trait-based approaches to exploring moss-associated N_2 fixation can complement and improve biogeochemical predictions as climate changes and are especially important to consider when employing proxies of the process.

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Author contribution statement SFM, JMP, and MCM conceived of the study that produced the data presented here, JEMS, HHM, MJ, SNM, SFD, JMP, and MCM performed research, JEMS and MCM analyzed data, JEMS wrote the manuscript with HHM, MJ, SNM, SFM, and MCM contributing to the manuscript.

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