

***Nostoc* Colonization Driven by *Sphagnum* Host Genetic Variation**

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ABSTRACT

Sphagnum-dominated peatlands sequester more carbon in temperate and boreal ecosystems than any other plant group. Carbon acquired in *Sphagnum* however is dependent on nitrogen availability, which is a limited nutrient in peatland ecosystems. *Sphagnum* derive much of their nitrogen budget from an intimate symbiosis with nitrogen-fixing diazotroph bacteria, but little is known about the factors mediating this symbiotic relationship. My research is guided by the overarching question: What are the genetic and physiological controls that shape *Sphagnum*-diazotroph symbiosis? Partnering with DOE JGI, we have (re)sequenced the genomes of 200 individuals from an *S. fallax* pedigree and the genome from an isolated diazotrophic cyanobacteria to create a QTL analysis to identify moss genes contributing to colonization. We hypothesize that there is variation in diazotroph colonization based on *Sphagnum fallax* genetic variation.

Here I present results from two independent experiments. First, a pH gradient experiment was conducted that demonstrated diazotroph colonization was mediated by pH demonstrating that symbiosis can have either a positive effect (mutualistic) or a negative effect (competitive) for one or both organisms. My results show that, at low pH, both organisms benefit in growth from symbiosis whereas cyanobacteria benefit more than moss at higher pH. Under pH conditions that fostered mutualism, I explored the genetic controls on the *Sphagnum*-diazotroph initiation by conducting a *Nostoc* colonization experiment. This study demonstrated that the amount of *Nostoc* colonization varied according to *Sphagnum fallax* genetic variation, that was illustrated according to a ranking of *Sphagnum* genotypes that had high *Nostoc* colonization to low *Nostoc* colonization. This was additionally supported by a recognized QTL analysis that related host genetic variation to variation in cyanobacterial colonization. The extreme *Sphagnum*

genotypes will serve as the basis for further studies examining the mechanisms underlying diazotroph colonization to provide an even better understanding of plant-microbe interactions, ecological genomics, and peatland carbon and nitrogen cycling.

Table of Contents

1 Introduction/Background	1
1.2 Study System	3
1.3 Cyanobacterial Symbiont	4
1.4 Symbiosis Predictions	4
2 Materials and Methods	7
2.1 Existing Genetic Resources.....	7
2.2 Symbiosis Growth Experiment.....	7
2.3 Co-Culture Colonization Experiment.....	9
2.4 DNA Isolation and Quantification	11
2.5 Primer Pair Establishment	11
2.6 PCR Verification	12
2.7 Absolute qPCR	12
2.8 Primer Comparison	13
2.9 Candidate Genes Underlying <i>Nostoc</i> Colonization in <i>S. fallax</i>	13
3 Results.....	15
3.1 Symbiosis Growth Experiment.....	15
3.2 Co-Culture Experiment	23
3.3 QTL Analysis.....	28
4 Discussion	32
References	35
Vita	43

List of Figures

Figure 1- S1 bog at Marcell Experimental Station in Minnesota depicting a hummock with <i>Sphagnum fallax</i> . Photo Credit: David Weston	3
Figure 2- Conceptual model depicting hypothesized association.	6
Figure 3- Conceptual design of example 12-well plate design of treatments.	9
Figure 4- Experimental plate design.....	11
Figure 5- Growth data of <i>Nostoc</i> alone and <i>Sphagnum</i> alone.....	17
Figure 6- Individual growth alone compared to growth in indirect contact with each other along the pH gradient.	17
Figure 7- Conceptual model depicting defining regions of the symbiotic interaction.	18
Figure 8- <i>Sphagnum</i> growth in symbiosis with <i>Nostoc</i> along the pH gradient.....	19
Figure 9- Regression of additive and non-additive effects in <i>Sphagnum</i> growth treatments with <i>Nostoc</i>	20
Figure 10- Diazotroph abundance using <i>nifH</i> primer pair.	25
Figure 11 - Diazotroph abundance using <i>POL</i> primer pair.	26
Figure 12- Overall abundance of diazotroph by treatment (control or <i>Nostoc</i> treated samples) using the <i>nifH</i> primer pair.	27
Figure 13- Overall abundance of diazotroph by treatment (control or <i>Nostoc</i> treated samples) using the <i>POL</i> primer pair.	27
Figure 14- Effect size by each <i>Sphagnum</i> genotype while using <i>nifH</i> primer pair.....	29
Figure 15- Effect size by each <i>Sphagnum</i> genotype while using <i>POL</i> primer pair.	29
Figure 16- QTL plot of colonization effect size using <i>nifH</i> primer pair.	30
Figure 17- QTL plot of colonization effect size using <i>POL</i> primer pair.	31

1 | Introduction/Background

Sphagnum peat mosses play a key role in ecosystem-level carbon and nutrient cycling. They are typically the dominant plant primary producers in peatland ecosystems (Kostka et al., 2016; M. R. Turetsky et al., 2012; Merritt R. Turetsky, Mack, Hollingsworth, & Harden, 2010) and accumulate large stores of dead organic plant material as recalcitrant peat (Gorham, 1991; Weston et al., 2018). Due to cold and anoxic conditions in low pH environments, the net production of *Sphagnum* mosses far surpasses decomposition (Vile et al., 2014). Over millennial timescales, this results in a tremendous amount of stored carbon as below ground peat (Gavazov et al., 2018), which is estimated to contribute around 30% of the world's soil carbon in just 3% of the global land surface (Clymo, 1987; Vile et al., 2014; Yu, Loisel, Brosseau, Beilman, & Hunt, 2010). Of all terrestrial ecosystems, peatlands have the highest carbon storage capacity per unit area (Stoneman, R. & Brooks, 1997; Worrall, Burt, Rowson, Warburton, & Adamson, 2009; Yu et al., 2010).

The ability for *Sphagnum* to acquire C through plant growth and production is dependent upon nitrogen availability, which is an extremely limiting nutrient in peatland ecosystems (Lindo, Nilsson, & Gundale, 2013; Weston et al., 2015). To help acquire N, *Sphagnum* peat mosses associate with N₂ fixing cyanobacteria (Basilier, 1980; Basilier, Granhall, & Stenstrom, 1978; Vile et al., 2014). From a plant productivity perspective, diazotrophic cyanobacteria are able to support *Sphagnum* growth of up to 35% with fixed nitrogen (Berg, Danielsson, & Svensson, 2013; Lindo et al., 2013; Weston et al., 2018). The extent and magnitude of *Sphagnum* symbiosis with N₂ fixing diazotrophic cyanobacteria directly influences peatland nutrient flux, carbon sequestration, and growth of vascular plants (Bay et al., 2013; M. R. Turetsky et al., 2012). Thus, the *Sphagnum* - cyanobacteria diazotroph association provides much needed

nitrogen to an otherwise nitrogen-limited ecosystem (Aerts, Wallen, & Malmer, 1992; Lamers, Bobbink, & Roelofs, 2000; Limpens & Berendse, 2003; Van Den Elzen et al., 2017).

Plant host genetics can influence the assembly of microbial associates (Bailey et al., 2005; Brachi et al., 2017; Kostka et al., 2016; Wagner et al., 2016) which can, in turn, affect plant health particularly under harsh environmental conditions (Bulgarelli, Schlaeppi, Spaepen, van Themaat, & Schulze-Lefert, 2013; Carrell et al., 2019). Plant-associated microbes can help defend against pathogens and herbivores (Christian, Whitaker, & Clay, 2015; Jones, Lawton, & Shachak, 1994) and contribute to plant phenotypic traits (Li et al., 2018). *Sphagnum* are known to host a diverse array of microbes with largely unknown functions (Kostka et al., 2016). *Sphagnum* is of high ecological importance since it is known to influence C and N nutrient cycling in peatlands, however the ecological impacts of *Sphagnum* associations with its microbiome are yet to be well characterized.

To better understand the ecological roles of host by microbiome interactions, the *Sphagnum* peat moss system is ideal to test because it has such a unique life cycle. *Sphagnum* exists primarily in the haploid gametophyte stage, with a short-lived diploid sporophyte stage (Weston et al., 2018). The dominant haploid life-cycle stage of mosses allow for genetic analyses to be simplified, as multiple genome copies (i.e. heterozygosity) can conceal allele specific expression (Weston et al., 2018). Consequently, F1 generations in controlled crosses can easily be used in trait mapping, this is not achievable for diploid, non-bryophyte systems (Weston et al., 2018). Importantly, the interactions at the plant-microbe level also can scale to influence ecosystem-level processes, as *Sphagnum* are ecosystem engineers that alter their surrounding habitat for their own benefit (Jones et al., 1994).

1.2 | Study System

As a part of the Spruce and Peatland Responses Under Changing Environments (SPRUCE) project, this study originated in the acidic ombrotrophic S1 bog at Marcell Experimental Station in Minnesota (Figure 1). The SPRUCE project has been providing valuable data pertaining to the ecology, hydrology, nutrient cycling and other aspects of peatlands since 1962 (Adams, 2004). Current research efforts are focused on determining the consequences of elevated CO₂ and warming on nutrient cycling. My current studies will contribute valuable perspectives on plant host - microbe interactions to the base of information available for the site. This contribution will enrich understanding of how variable environments influence nutrient cycling by accounting for often neglected plant-associated microbes that can be a major factor on how ecosystems respond to environmental change.



Figure 1- S1 bog at Marcell Experimental Station in Minnesota depicting a hummock with Sphagnum fallax. Photo Credit: David Weston

1.3 | Cyanobacterial Symbiont

It is well understood that Cyanobacteria associate with *Sphagnum spp.* peat mosses (GRANHALL & HOFSTEN, 1976; Kostka et al., 2016) but the ubiquity of the association and impact on ecosystem level processes has not been thoroughly characterized. Prior research has shown that certain cyanobacteria are able to form symbioses with plants, causing a differentiation of motile filaments known as hormogonia into specialized cells known as heterocysts that are able to fix atmospheric nitrogen into a usable form (e.g., ammonium) for plants (Kumar, Mella-Herrera, & Golden, 2010; Meeks & Elhai, 2002). Many cyanobacteria are able to fix nitrogen (diazotroph) using the nitrogenase enzyme (Fay, 1992; Kumar et al., 2010; Meeks & Elhai, 2002; Robson & Postgate, 1980). Nitrogenase, encoded by *nif* genes, catalyzes the reduction of atmospheric N_2 into ammonium or nitrate that is then made available to the host plant (Ininbergs, Bay, Rasmussen, Wardle, & Nilsson, 2011). In the study system of *Pleurozium schreberi*, it has been suggested that in exchange for N as ammonium, *P. schreberi* provides C in the form of carbohydrates to the cyanobacterial symbiont (Rousk, Jones, & DeLuca, 2013). Similarly, the *Sphagnum* microbiome contains *nif* genes (Leppänen, Rissanen, & Tirola, 2015) and has been shown to fix N_2 demonstrating that *Sphagnum* associates strongly with diazotrophs. Although nitrogen fixation in mosses is believed to be controlled by many environmental factors (Leppänen et al., 2015), such as light and temperature, it is the extent of *Nostoc* colonization that is thought to predominantly influence *Sphagnum* production and carbon sequestration (Gentili, Nilsson, Zackrisson, DeLuca, & Sellstedt, 2005).

1.4 | Symbiosis Predictions

The role of how plant-associated microbes, especially mutualists, impact a species niche is not well understood (Peay, 2016). The concept known as the n-dimensional hypervolume is an

interpretation of the fundamental niche that explains there are necessary environmental factors that a species must occupy to survive (Hutchinson, 1957). Further, the interactions of a species must be characterized by multiple biotic and abiotic factors that confine the health and growth of a species (Hutchinson, 1957). From this interpretation by Hutchinson, I believe to better comprehend the mechanisms underlying the interactions for both host and microbial associates, one must explore the host genetics and environmental factors associated with the fundamental niche of each organism in isolation of each other to fully estimate how the interaction may affect each niche.

Sphagnum and its symbiotic diazotrophs are known to have a mutualistic relationship, but the mechanisms of the association are not well defined. Since diazotrophs are known to convert atmospheric nitrogen into N rich molecules such as ammonia, it is hypothesized that *S. fallax* receives nitrogen in the fixed form of ammonia or nitrate from *Nostoc spp.* and in return *S. fallax* provides the diazotroph with carbohydrates, protection and shelter from the extreme peat bog environment (Bay et al., 2013; Rousk et al., 2013; Warshan et al., 2017). Figure 2 depicts how the association is known to occur. To explore the mechanisms of this association; the questions being posed here are, does the less explored environmental factor of pH influence *Nostoc* colonization in *Sphagnum* and does *Sphagnum* host genetic variation influence the magnitude of *Nostoc* symbiosis?

We hypothesize that the outcome of *Sphagnum-Nostoc* symbiosis will vary along an aqueous pH gradient and *Sphagnum* hosts exhibit genetic variation in internal chemical environments which, in turn, influences diazotroph colonization. The objective of this study is to determine what environmental conditions, such as pH, are optimal for growth of *Sphagnum* and *Nostoc* in symbiosis and to then measure diazotroph abundance across a genetically diverse

Sphagnum population at an optimal pH for the interaction to occur. I have tested this hypothesis by examining diazotroph colonization according to genetic variation in a population of *S. fallax* half siblings in order to gain insight into the role of host genetics in mediating *Sphagnum*-diazotroph interactions. The goal of this study is to better characterize the host genetics and influence of pH conditions that are factors in defining this ecologically relevant interaction.

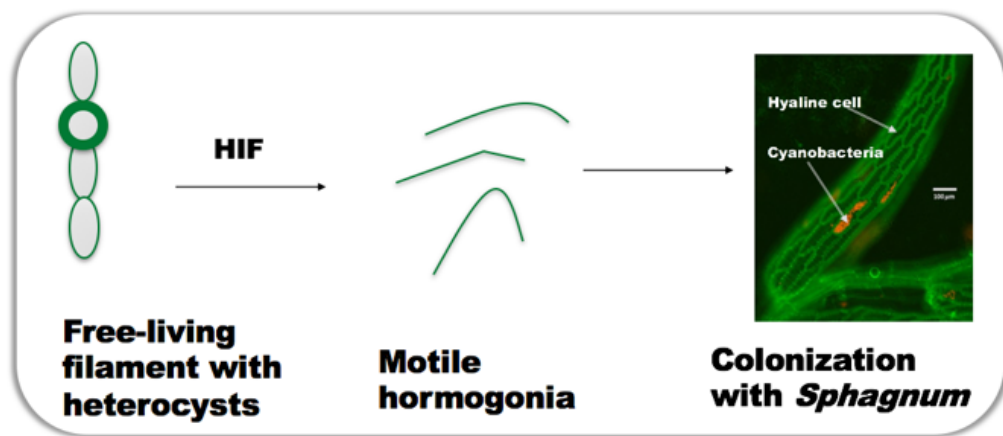


Figure 2- Conceptual model depicting hypothesized association.

Modified from Weston et al. 2015, diazotrophic cyanobacteria must sense the plant host through a secreted hormogonia-inducing factor, HIF, and move to the moss host. The hormogonia is a motile form of the cyanobacteria that occurs through differentiation of vegetative cells. Then the cyanobacteria can colonize inside the hyaline cells endophytically or on the cell surface epiphytically.

2 | Materials and Methods

2.1 | Existing Genetic Resources

A *S. fallax* mother containing a capsulated sporophyte was collected on July 15, 2012 and was transported from the S1 bog at Marcell experimental station in Minnesota and received at Oak Ridge National Laboratory. The sporophyte was then surface sterilized and ruptured into a sterile 1.5 ml microcentrifuge tube to create the F1 progeny of half siblings. A total of 184 *S. fallax* individuals were previously isolated and sequenced to provide an established genetic linkage map of this population to aid in gene to trait experiments (Weston et al., 2018).

2.2 | Symbiosis Growth Experiment

A co-culture experiment was conducted to assess the effects of *Sphagnum* and *Nostoc* growth over a pH gradient. The experiment was implemented by using 12-well Falcon plates and three growth combinations including *Sphagnum* grown alone (control), *Nostoc* grown alone (control), or *Sphagnum* grown with cyanobacteria. Six replicates of *Sphagnum fallax* and *Nostoc* were used per pH treatment. For the *Sphagnum* with cyanobacteria treatments, *Sphagnum* was grown above an 8.0 μm pore size filter with *Nostoc* beneath. This ensured that measures were taken of intracellular colonization by *Nostoc* because the filter only allows motile hormogonia to pass and colonize *S. fallax* hyaline cells. Each 12-well plate consisted of a BG-11 growth media containing no nitrogen or carbohydrate source. The pH levels tested were 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5. The contents of individual cells incubated for 6 weeks at a temperature of 25°C/18°C (day/night) and light strength of $\sim 110 \mu\text{mol m}^{-2} \text{s}^{-1}$ at a 12-hour photoperiod. Figure 3 shows a depiction of an example plate design containing pH treatments of 3.5 and 4.5. An ANOVA statistical analysis was used to measure variation among treatments to show significance in growth effects along the pH gradient.

It is known that *Nostoc* survival in terrestrial ecosystems are in part dependent upon its interaction with organisms such as mosses (Dodds, Gudder, & Mollenhauer, 1995), while *Sphagnum* are known to be ecosystem engineers, having the ability to manipulate their habitat for their own benefit (Jones et al., 1994). Knowing the history of *Sphagnum* and *Nostoc* leads me to conclude that they potentially occupy differing fundamental niches. I believe that past research has not yet fully taken into account the impacts of microbial interactions within *Sphagnum* and how those interactions may play a significant role into constructing what is known to be the fundamental niche for *Sphagnum*. Therefore, a conceptual model of how the symbiotic interaction can be defined based on results of the average *Sphagnum* growth patterns in isolation without *Nostoc* was constructed. This conceptual model will be used as a framework to understand how the symbiotic interaction may alter along an aqueous pH gradient.

To evaluate whether *Sphagnum* modifies its surrounding environment, we conducted a similar supplementary study where *Sphagnum* induced pH change of liquid media was monitored for 3 weeks of incubation. The change in pH of liquid media was based on 4 replicates of the following treatments; *S. fallax* control, *Nostoc* control, or *S. fallax* in direct contact with *Nostoc*. In this experiment, 4 replicates of each treatment were grown individually and in direct contact with each other in wells containing a BG-11 no nitrogen media at a pH of 3.5, 5.5, and 8.5 for 3 weeks in 12-well Falcon plates. The difference in set up of this supplemental work and the pH growth experiment was that *Nostoc* and *Sphagnum* were not separated by a filter, thus allowing for *Sphagnum* to be in direct contact with mature *Nostoc*. We measured the change in pH of the liquid media by using a pH electrode at the start of the experiment and compared that to the pH at the end of the experimental 3 weeks. A Welch's two-sample t-test was used to infer statistical differences between the initial pH and the final pH for each treatment. This experiment will

determine if either *Sphagnum* or *Nostoc* have the ability to change their environmental conditions when grown alone and how that may change when they are interacting directly.

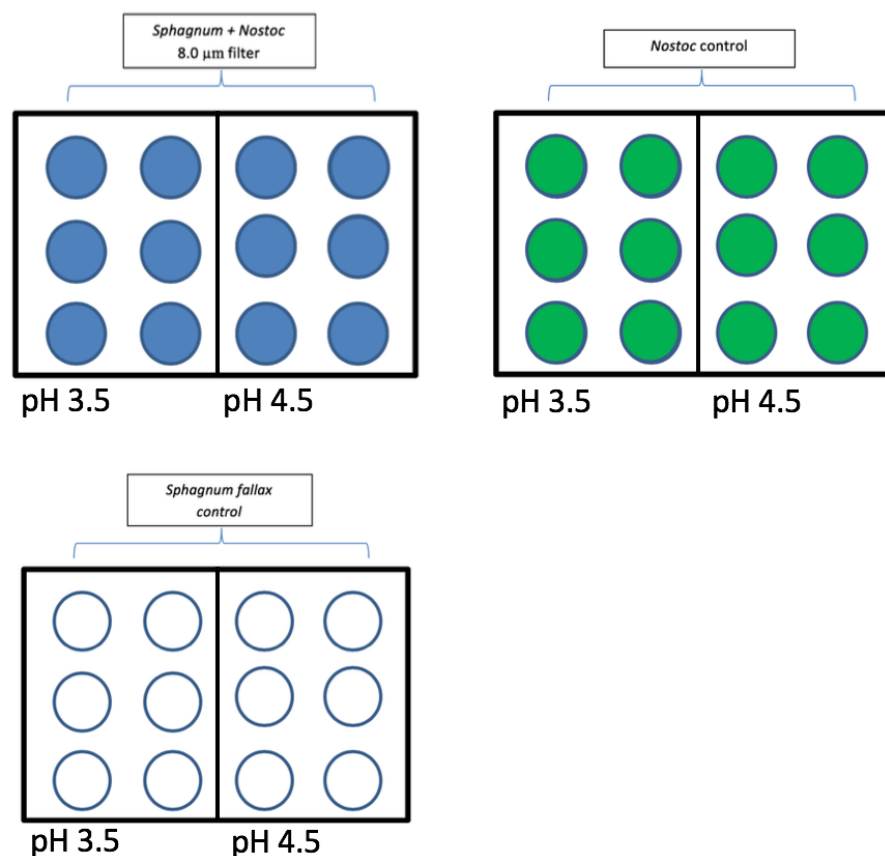


Figure 3- Conceptual design of example 12-well plate design of treatments.

Conceptual design illustrating an example plate set up shown containing pH 3.5 and 4.5. Each plate contained 1 treatment across a pH gradient that ranged from 3.5 to 8.5. The plates contained 6 replicates of the specified treatment (*Sphagnum* + *Nostoc* with a filter separation, *Nostoc* control, or *Sphagnum* control) across each pH to allow for growth of both individuals in isolation and in symbiosis to be observed over a 6-week time period.

2.3 | Co-Culture Colonization Experiment

To assess the influence of host genetic variation on *Nostoc* colonization, a co-culture experiment was conducted using 12-well Falcon plates (Figure 4). Each treatment well within the

plate contained an 8.0 μm pore size filter to exclude physical association of *S. fallax* with mature *Nostoc*, however the filter allowed motile immature *Nostoc* hormogonia to pass (Bay et al., 2013). This interaction was visualized through light microscopy to ensure that hormogonia could pass through the filter and were colonizing within the hyaline cell, the filter was investigated on a tabletop electron microscope. The contents of individual cells incubated for one week at a temperature of 25°C/18°C (day/night) with light intensity set to $\sim 110 \mu\text{mol m}^{-2} \text{s}^{-1}$ on a 12-hour photoperiod. Figure 4 depicts the experimental set up. Each 12-well plate consisted of 3 *S. fallax* genotypes with 2 technical replicates of each *S. fallax* genotype. Technical replicates were placed either on top of an 8.0 μm pore diameter filter insert as a treatment or directly in the designated well as a control. Below the filter, a 2 ml solution containing either 0.1 grams of *Nostoc* (treatment) or no filter added medium alone (control). The *Nostoc* stock culture was grown in BG-11 medium without nitrogen at pH 8.0. Prior to incubations, the pH was adjusted to 4.5 to facilitate colonization. BG-11 media consists of all necessary nutrients for cyanobacteria and plant growth but does not contain nitrogen or a carbohydrate source.

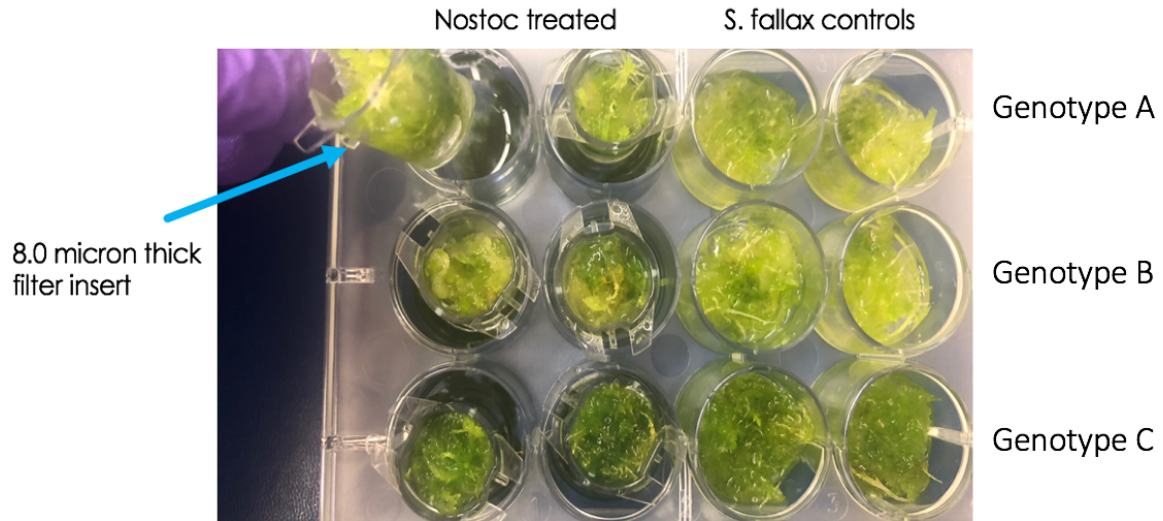


Figure 4- Experimental plate design.

Illustration of 3 *S. fallax* genotypes in BG-11 no nitrogen media set to a pH of 4.5. 110 genotypes were cultured and had 2 technical replicates for control samples (no *Nostoc*) and for the treated samples (*Nostoc* separated by filter).

2.4 | DNA Isolation and Quantification

Sphagnum tissue was harvested, and flash frozen prior to DNA isolation. Each individual was ground into a powder by mortar and pestle in liquid nitrogen and tissue was measured out to ~1 gram per sample. DNA isolations were conducted via a modified CTAB (Hexadecyltrimethylammonium bromide) method (Doyle, J.L and Doyle, 1990). The remaining pellet of DNA was then resuspended in 60 μ l of nuclease free water overnight so that the pellet was completely in solution. The quantification and quality of DNA was checked using nanodrop, qubit, and gel electrophoresis.

2.5 | Primer Pair Establishment

Primer pairs from previous research regarding amplification of the *NifH* gene region were tested to evaluate the presence of *Nostoc* colonization across *S. fallax* genotypes. Primers

were chosen based on the NifH gene region, which is found in the *Nostoc* genome and in other diazotroph genomes. 2 commonly used primer pair sets were tested via PCR on a subset of samples to optimize conditions for qPCR as follows, POLF: 5'-TGCGAYCCSAARGCBGACTC - 3'/POLR: 5'-ATSGCCATCATYTCRCCGGA - 3' (Poly et al, 2002; Carrell et al., 2019) and nifH-F: 5' – AAAGGYGGWATCGGYAARTCCACCAC – 3'/nifH-R: 5' - TTGTTSGCSGCRTACATSGCCATCAT – 3' (Rösch, Mergel, & Bothe, 2002) . The POL primer set amplifies PCR products of NifH gene fragments at 360 base pairs (bp) and the nifH primer set amplifies PCR product of NifH gene fragments at 457 bp.

2.6 | PCR Verification

PCR was conducted using Thermo Scientific DreamTaq Hot Start PCR Master Mix (2X) 25 µl reactions on a subset of samples using the POL and nifH primer pairs. Both primer pairs were optimized to proper thermal cycling conditions via PCR testing. Presence/absence of nifH was detected by analyzing PCR products on agarose gels stained with ethidium bromide at 100 volts for 45 minutes in a 1X TAE buffer. Gel electrophoresis visualized results by separating DNA fragments by base pair (bp) size to obtain an amplicon band from the primer-targeted region.

2.7 | Absolute qPCR

Primers listed above were used to quantify the total abundance of nifH gene copy numbers across each *S. fallax* individual. Each *S. fallax* individual gDNA concentration was diluted to a working stock at a concentration of 10 ng/µl. A standard curve was created by making serial dilutions (1:5) of the standard of known *Nostoc* gDNA concentrations. qPCR was conducted on an Applied Biosystems StepOnePlus Real-Time PCR System Thermal Cycling Block using BIO-RAD iTaq Universal SYBR Green Supermix (2X) at 20 µl reactions in 96 well

plates. Conditions for qPCR using both primers were optimized to 95° for 15 minutes, 94° for 30 seconds, 63° for 45 seconds, melt curve at 95° for 15 seconds, 60° for 1 minute, and 95° for 15 seconds. qPCR was performed on 110 *Sphagnum* individuals that contained 3 technical replicates for more efficiency in results. Diazotroph abundance was measured by calculating gene copy number and was analyzed based on a standard curve. First, the gene copy number of known standards of *Nostoc* gDNA was calculated. Then, a standard curve efficiency graph was created by making serial dilutions of known *Nostoc* gDNA concentration and plotting the average cycle threshold values (Ct) on the y-axis by the logarithm of the quantity of gene copy number on the x-axis. Finally, based on the standard curve calculations and average Ct values of technical replicates of genotypes, precise calculations were made to measure the abundance of *Nostoc* per gram of *S. fallax* that it was extracted from.

2.8 | Primer Comparison

Each experimental sample was used in qPCR to measure the total abundance of diazotroph gene copy number per *Sphagnum* genotype by utilizing results from 2 different primer pair sets. Though both have been used in previous research to measure nifH activity, qPCR was conducted with POLF/POLR and nifHF/nifHR to characterize possible differences between each primer pair. The data for both primer sets were filtered to only include genotypes that exhibited no amplification of nifH fragments in control samples. A spearman rank correlation was used to test the relationship between colonization abundance as estimated from the two primer sets among identical genotypes.

2.9 | Candidate Genes Underlying *Nostoc* Colonization in *S. fallax*

Absolute qPCR results were analyzed to obtain effect sizes for each genotype and those effect sizes were plotted by genotype to determine high or low colonizing genotypes. Effect size

was measured by $((\text{treated copy number} - \text{control copy number}) / \text{Average}(\text{treated copy number} - \text{control copy number}))$. Filtering of data, previously mentioned, lowered the sample size significantly to only 58 genotypes assayed with nifHF/nifHR primers and 21 genotypes assayed with POLF/POLR primers. This demonstrated that the genotype with the lowest effect size was P4A3 (0.611) while the high extreme genotype was P6B2 (5.68), according to the POL primer pair. But according to the nifH primer pair, the low extreme genotype was P17B3 (0.0050) while the high extreme genotype was P15C1 (3.90).

Colonization effect sizes were then used as a phenotype in a Quantitative Trait Loci (QTL) analysis to determine the specific gene regions of the *Sphagnum* genome that may be correlated to diazotroph colonization. QTL analyses were conducted by using the phenotype estimates of both POL and nifH primer sets. 2 different statistical algorithms were used to test the strength of evidence that there is presence of the phenotype (colonization effect size) at a particular location on the genome. These genotypes and other similar genotypes will be utilized in further experimentation to estimate what percentage of diazotroph colonization within *Sphagnum* hyaline cells is fixing nitrogen.

3 | Results

3.1 | Symbiosis Growth Experiment

Sphagnum and *Nostoc* require differing environmental conditions to grow and survive, but the conditions for the association to occur are not yet defined. A pH gradient experiment was implemented to assess whether pH influences the *Sphagnum-Nostoc* association, focusing on measures of growth for both *Sphagnum fallax* and *Nostoc*. Growth was determined according to a measure of delta wet weight in grams (initial – final growth weight). Figure 5 shows the individual growth patterns of *Nostoc* and *Sphagnum* grown alone across 6 pH levels. The growth of both organisms in isolation of each other have individual responses to pH. *Nostoc* grown in isolation prefers more basic pH levels while *Sphagnum* grown in isolation has no significant response to the pH gradient. Based on an ANOVA, I found that the growth of *Nostoc* in isolation had a significant difference (p-value = $8.02e^{-07}$) across the pH gradient, however the growth of *Sphagnum* in isolation showed no significant difference (p-value = 0.506) across the pH gradient.

Sphagnum growth in indirect contact with *Nostoc* has a noticeably obvious shift in growth along the pH gradient as compared to the *Sphagnum* growth alone, however this difference is not significant (p-value = 0.351) (Figure 6). *Nostoc* growth while in indirect contact with *Sphagnum* was only slightly higher than *Nostoc* growth in isolation of *Sphagnum* (Figure 6), but the change in growth was nonetheless significant, based on statistical analysis from an ANOVA (p-value = 0.0153).

Based on the average *Sphagnum* growth pattern in isolation (Figure 5), I present a conceptual model of how the symbiosis will be characterized (Figure 7). If the association is beneficial to the moss host, the average growth for *Sphagnum* in isolation will be greater than growth that occurs when in contact with the cyanobacterial symbiont. Alternatively, if the relationship is competitive or antagonistic, growth in symbiosis will be lower than the average

growth of *Sphagnum* in isolation. These relationships are depicted as a conceptual model in Figure 7. Results of an ANOVA revealed that there is a significant difference (p-value = 0.002) between growth of *Sphagnum* in symbiosis with *Nostoc* across the pH gradient (Figure 8). At acidic pH levels such as 3.5 and 4.5, the symbiosis is more beneficial for *Sphagnum* growth (Figure 8). At basic pH levels such as 7.5 and 8.5, *Sphagnum* perform better alone than with *Nostoc*.

Legacy of past work allows for inferences to be made about the individual growth patterns of these organisms based on pH preference. This *Sphagnum-Nostoc* interaction cannot be estimated correctly based on the additive effects from the sum of the individual growth effects (*Sphagnum* control plus *Nostoc* control), the non-additive effects of growth in *Sphagnum* while in symbiosis suggest a better estimate into the mechanisms that drive the interaction. When looking at the actual data, we do not see the same growth pattern in the sum of individual growth effects across the pH gradient that we see while in indirect contact (Figure 9). The additive effects show a mutualistic relationship across the pH gradient while the non-additive effects show the mutualistic relationship relies on the pH environment. Table 1 shows the statistical significance from an ANOVA among the variation in growth treatments across pH. Based on this experimentation and previous work, we chose to execute further experiments at a pH of 4.5.

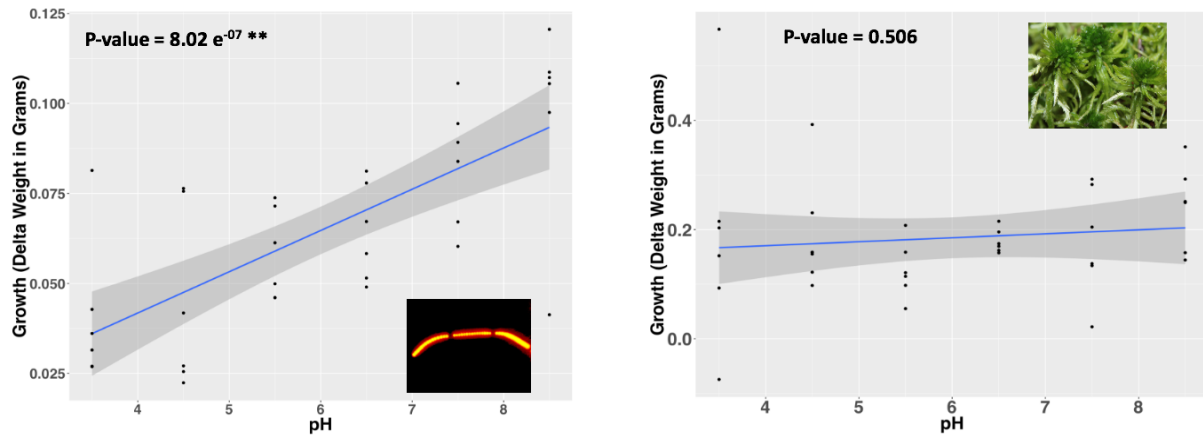


Figure 5- Growth data of *Nostoc* alone and *Sphagnum* alone

Growth of both organisms alone across 6 pH conditions over a period of 6 weeks. There is considerable growth variation across pH in *Nostoc* when grown alone (Left). *Sphagnum* did not exhibit variation in response to changing pH conditions (Right).

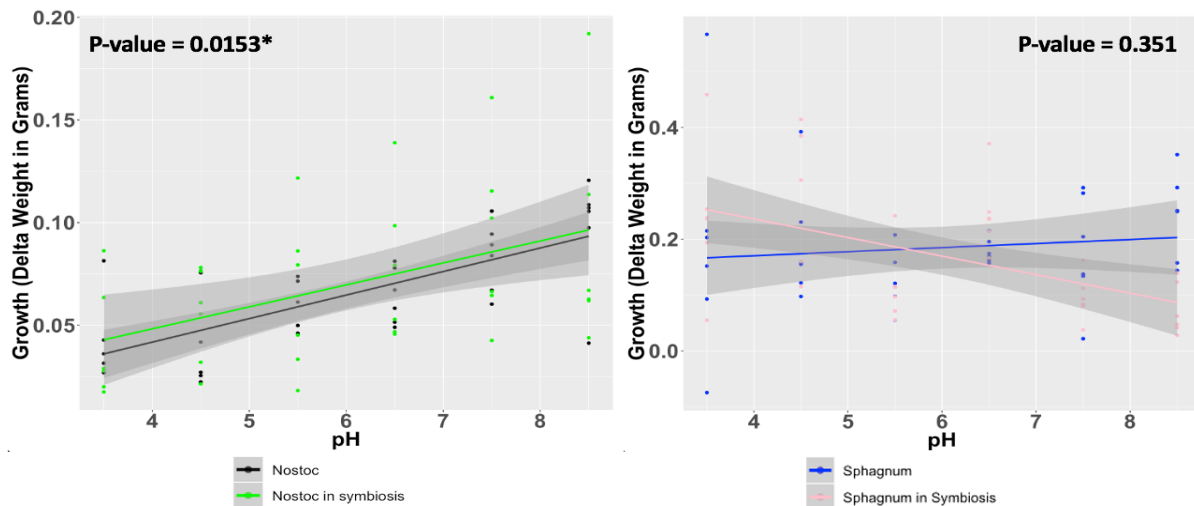


Figure 6- Individual growth alone compared to growth in indirect contact with each other along the pH gradient.

Nostoc growth alone in response to pH (black line) and when in indirect contact with *Sphagnum* along pH gradient (green line). There is a significant difference in *Nostoc* growth alone when compared to growth while in indirect contact across pH (p-value = 0.005***) (Left). *Sphagnum* growth alone in response to pH (blue line) and when in indirect contact with *Nostoc* along pH gradient (pink line). Visually there is a different pattern in growth of *Sphagnum* alone when compared to growth while in indirect contact with *Nostoc* across pH, however the difference is not significant (p-value = 0.351) (Right).

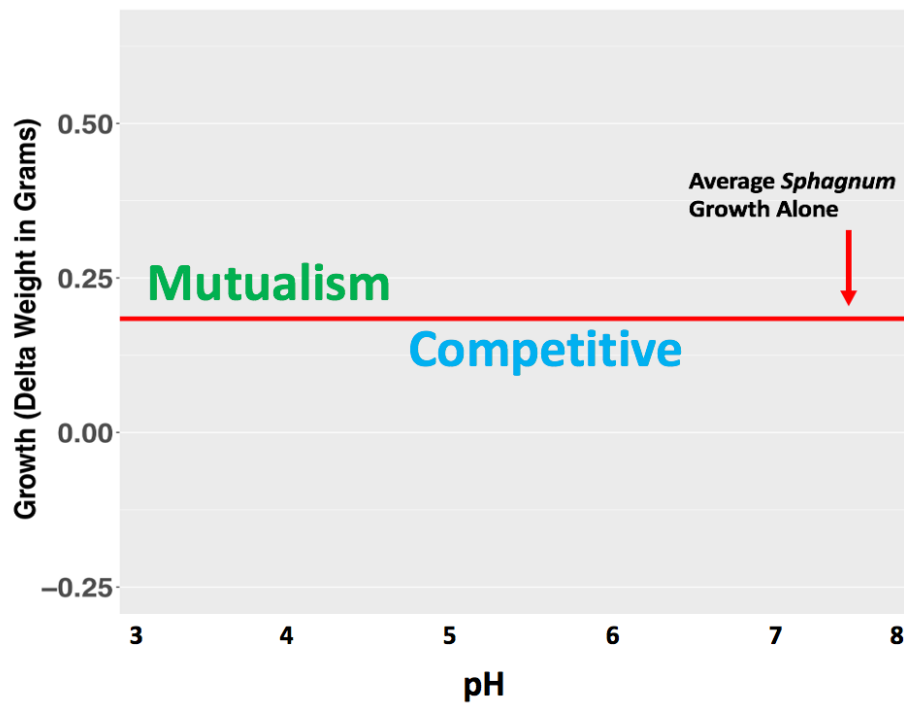


Figure 7- Conceptual model depicting defining regions of the symbiotic interaction.

Red line depicts the y-intercept as the mean *Sphagnum* growth with no symbiont across pH. To be considered mutualistic, the growth must surpass that line, otherwise it is viewed as a competitive effect.

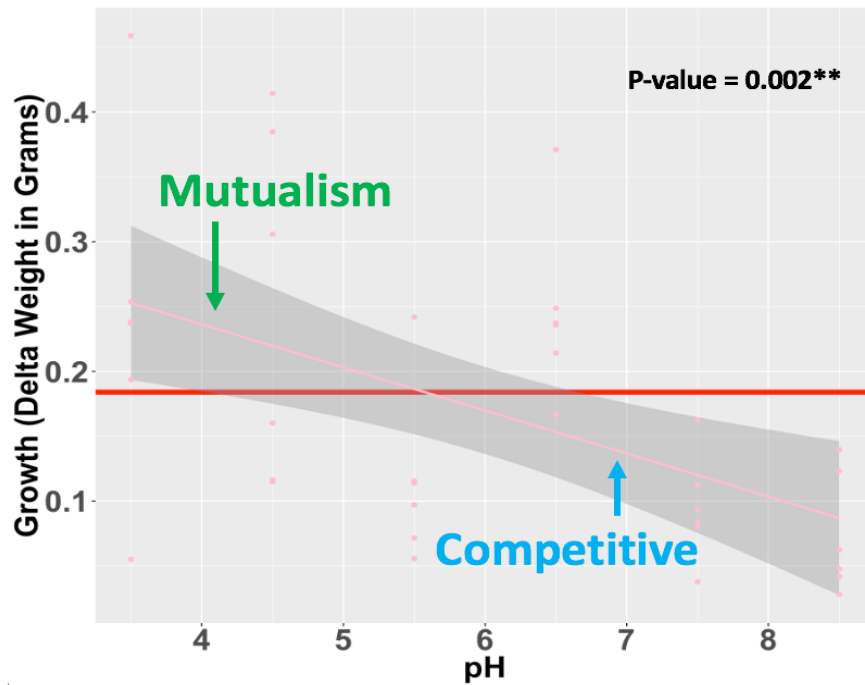


Figure 8- *Sphagnum* growth in symbiosis with *Nostoc* along the pH gradient.

Sphagnum host growth while in symbiosis with *Nostoc* in response to pH alone (pink line). The mean *Sphagnum* growth alone (red line). There is a significant difference in growth while in symbiosis across pH (p-value = 0.002**). Mutualism occurs at acidic pH levels while a competitive interaction occurs at basic pH levels.

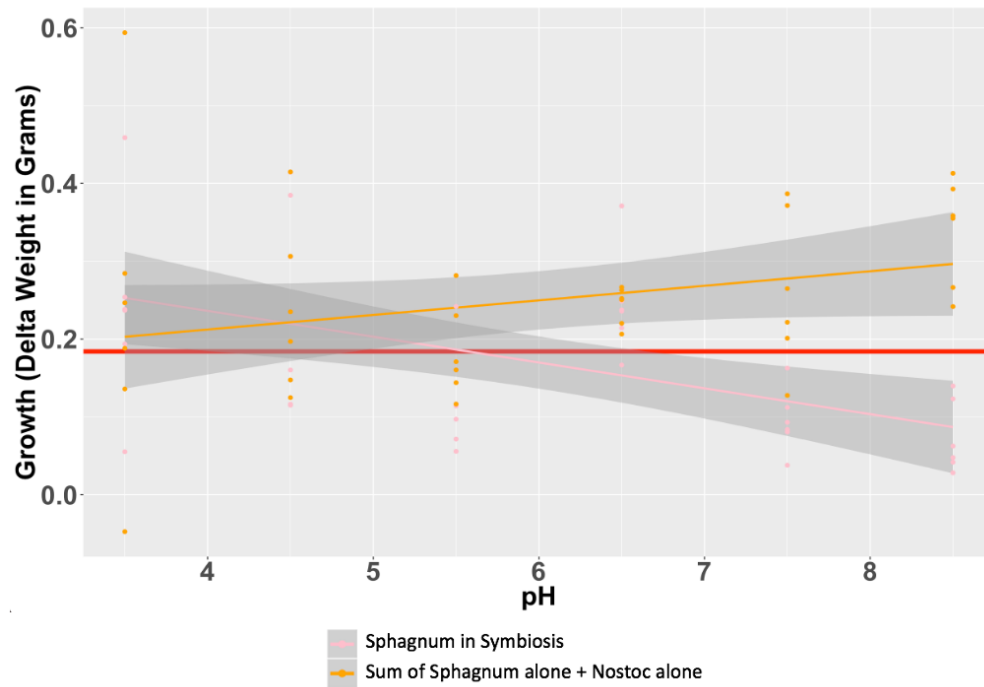


Figure 9- Regression of additive and non-additive effects in *Sphagnum* growth treatments with *Nostoc*.

The sum of *Sphagnum* growth alone added to *Nostoc* growth alone (orange line) depict additive effects on biomass while *Sphagnum* growth in symbiosis (pink line) suggests that niche dimensions are being determined by pH. Shadow effects are depicting 95% confidence intervals.

Table 1 Variation of growth treatments across pH to show how significant growth in symbiosis can be compared to growth alone and how that varies across a pH gradient. Note: ** $p < .001$, *** $p < 0$

<i>Variation of Growth</i>	<i>p-value</i>	<i>F-value</i>	<i>Sum of Squares</i>
<i>Across pH</i>			
<i>Nostoc Alone</i>	8.02×10^{-07} ***	36.28	54.21
<i>Sphagnum Alone</i>	0.506	0.451	1.38
<i>Nostoc Alone by</i>	0.0153*	6.518	0.004298
<i>Growth in</i>			
<i>Symbiosis</i>			
<i>Sphagnum Alone by</i>	0.351	0.893	0.0108
<i>Growth in</i>			
<i>Symbiosis</i>			
<i>Nostoc in Symbiosis</i>	0.00514**	8.947	21.87
<i>Sphagnum in</i>	0.00158**	11.79	27.04
<i>Symbiosis</i>			
<i>Sum of Sphagnum</i>	0.0924	2.998	0.0368
<i>and Nostoc Growth</i>			
<i>Alone</i>			

To assess whether *Sphagnum* are manipulating their surrounding environment for their own benefit, a supplementary study monitored the change in pH of liquid media occupied by either *Sphagnum*, *Nostoc*, or, *Sphagnum* in direct contact with *Nostoc* in 3 initial pH conditions for 3 weeks. Table 2 shows the change in pH based on those treatments. From a Welch's two-sample t-test, for all treatments, there was significant change from the initial pH to the final pH (Table 2). Overall, the *Sphagnum* control samples imposed a significant change to more acidic pH when at the basic pH of 8.5 and the *Nostoc* control samples imposed a significant change from the acidic 3.5 pH to a more basic pH. While in direct contact with each other; however, the change in pH was significant but appears to level off to a more neutral pH ranging from about 6 to 7.

Table 2- Change in pH based on individual composition.

Treatment	Initial pH	Average Final pH	Standard Deviation	p-value
Fallax Control	3.5	3.7	0.05	0.0029
Fallax Control	5.5	6.1	0.14	0.0034
Fallax Control	8.5	7.2	0.34	0.0045
Fallax in Direct Contact with <i>Nostoc</i>	3.5	5.7	0.2	0.00021
Fallax in Direct Contact with <i>Nostoc</i>	5.5	6.1	0.24	0.0191
Fallax in Direct Contact with <i>Nostoc</i>	8.5	6.7	0.1	4.342e- 05
<i>Nostoc</i> Control	3.5	6.0	0.21	0.00015
<i>Nostoc</i> Control	5.5	6.5	0.18	0.00133
<i>Nostoc</i> Control	8.5	7.0	0.11	0.00010

3.2 | Co-Culture Experiment

I estimated cyanobacteria abundance within the host using qPCR to assess whether there is genetic variation within the *Sphagnum fallax* population for cyanobacterial colonization. I found that *Sphagnum* genotypes inoculated with *Nostoc* have a higher abundance of diazotroph than corresponding control samples of *Sphagnum* alone (Figure 10 and Figure 11). There was a significant difference in variation of treatment across genotype for the *nifH* primer set (p-value =

0.013*), whereas differences in colonization assayed with the POL primer set were not significant (p-value = 0.926). Treated samples (*Nostoc* inoculated) were ranked from high to low diazotroph abundance according to both the nifH and POL primer set assays (Figure 10 and Figure 11).

Comparing the primer pairs, the correlation coefficient between the treated values was 0.48 and had a significant p-value of 0.048, between the control values was 0.20 with a non-significant p-value of 0.447, suggesting that there is a positive correlation between the treated values while there is only a non-significant weak correlation between control values. Overall analysis from ANOVA's show there was a significant difference found between overall treatments based on the nifH primer set assay (p-value = 5.62×10^{-13}) (Figure 12). There was also a significant though weaker difference between overall treatments based on POL primer set assay (p-value = 0.0361) (Figure 13), where treated genotypes have a higher average copy number of diazotroph abundance than control genotypes. This was corroborated with light microscopy; *Nostoc* was found living inside treated *Sphagnum* hyaline cells after 1 week of incubation. The filter was also explored with a tabletop electron microscope after 1 week of incubation; filamentous *Nostoc* was found on both sides of the filter.

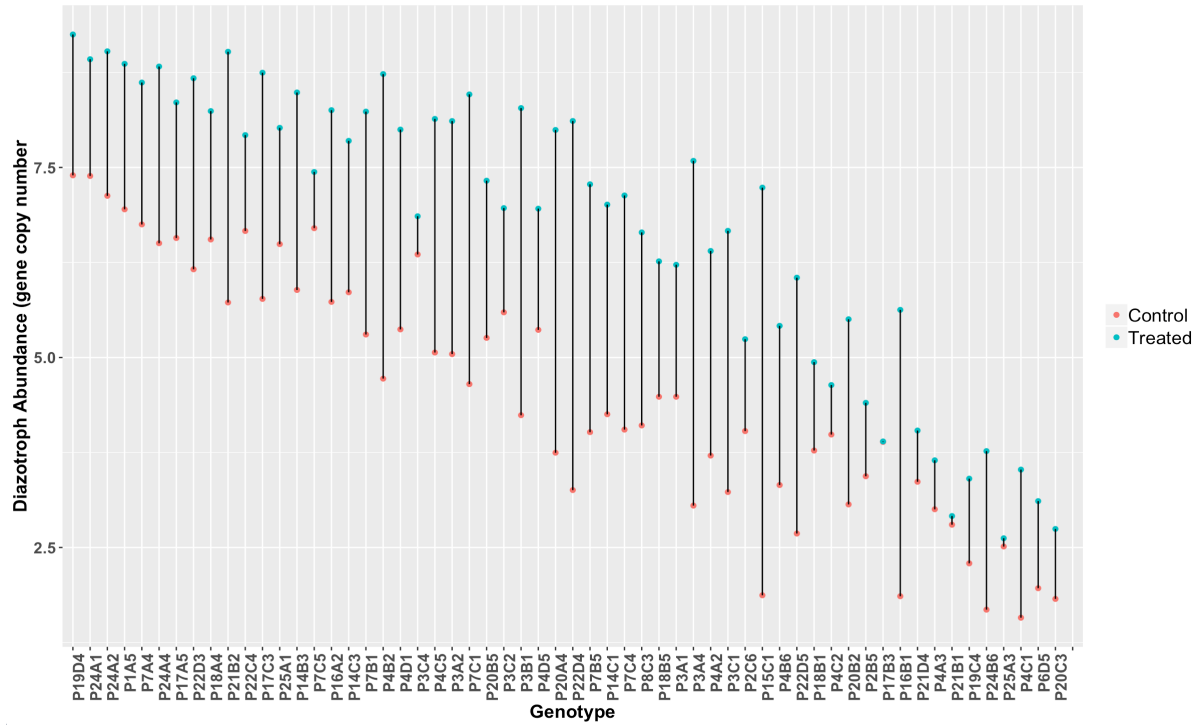


Figure 10- Diazotroph abundance using *nifH* primer pair.

Depiction of diazotroph abundance according to gene copy number values using the *nifH* primers for treated (*Nostoc* inoculated) and control (*Sphagnum* without inoculation) conditions across *Sphagnum* genotypes ranked by colonization abundance. There was a significant difference in variation according to treatment across genotypes (p-value = 0.013*).

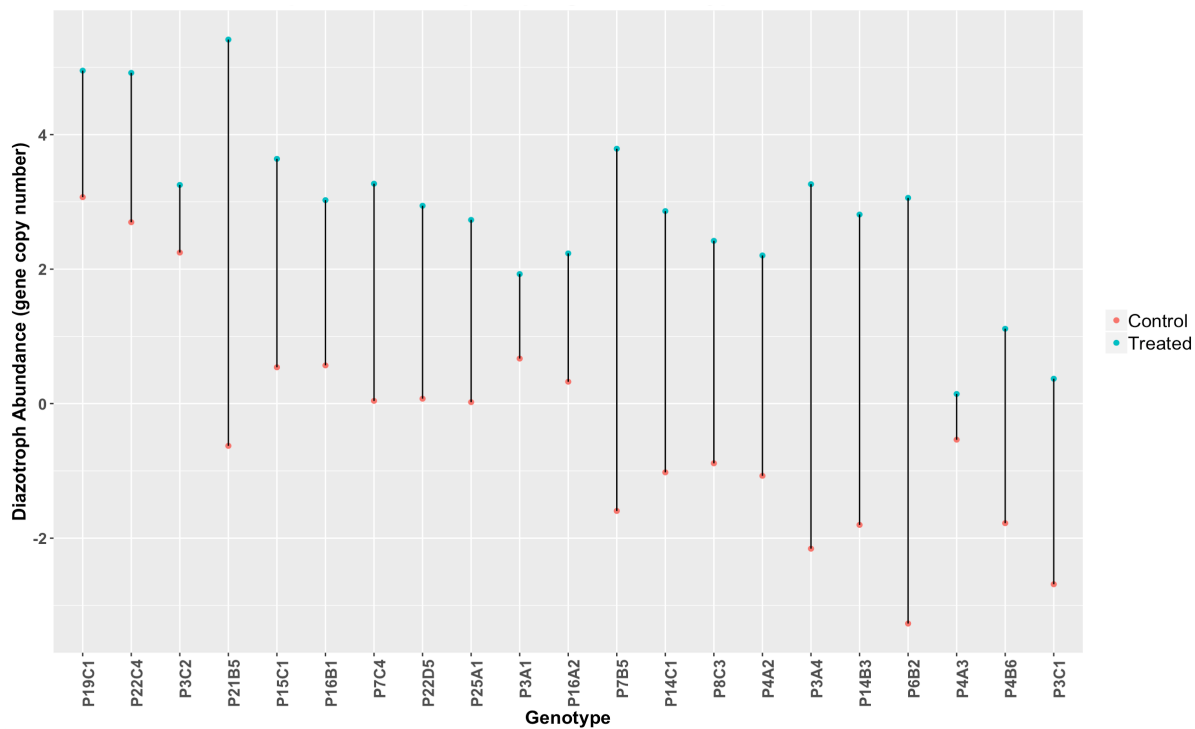


Figure 11 - Diazotroph abundance using POL primer pair.

Depiction of diazotroph abundance according to gene copy number values using POL primers for treated (*Nostoc* inoculated) and control (*Sphagnum* without inoculation) conditions across *Sphagnum* genotypes ranked by colonization abundance. The difference in variation according to treatment across genotypes was not significant (p-value = 0.926).

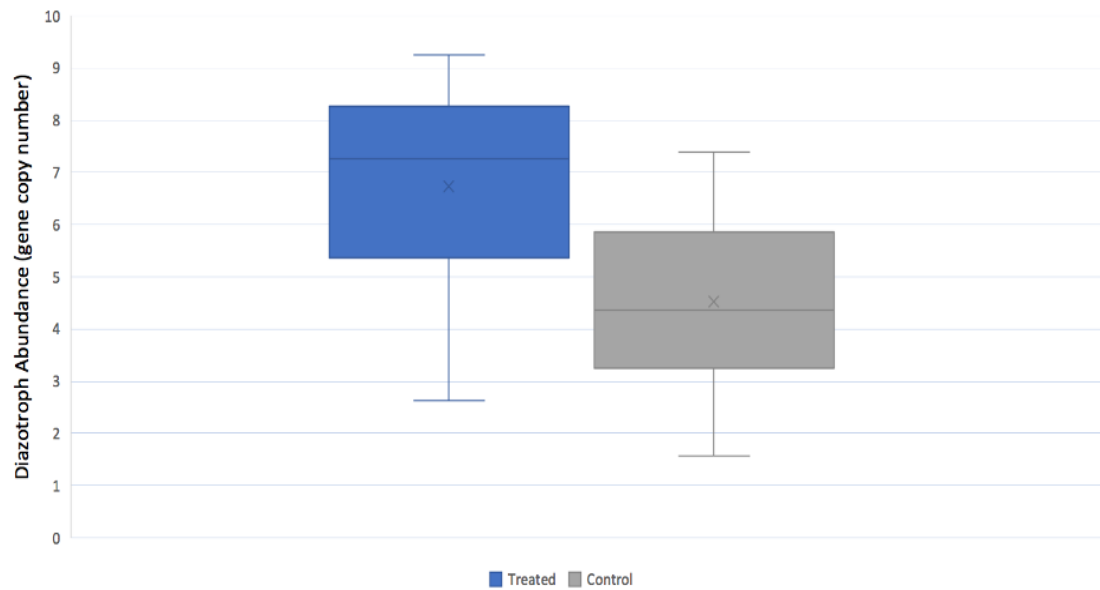


Figure 12- Overall abundance of diazotroph by treatment (control or *Nostoc* treated samples) using the *nifH* primer pair.

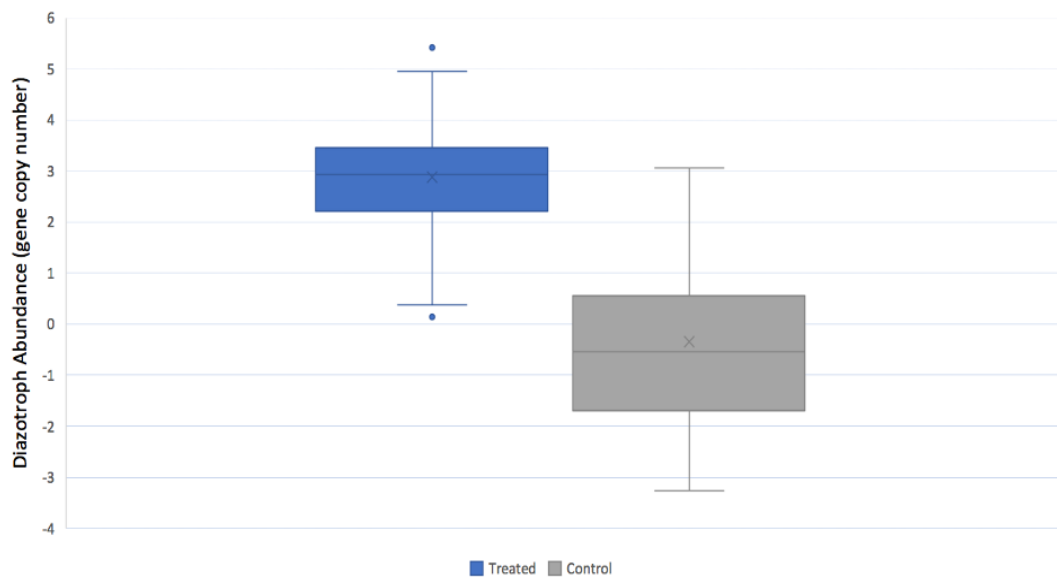


Figure 13- Overall abundance of diazotroph by treatment (control or *Nostoc* treated samples) using the *POL* primer pair.

3.3 | QTL Analysis

To evaluate if there is a genetic basis to the mutualistic interaction, a QTL analysis was performed to relate host genetic variation to variation in cyanobacterial colonization. To do this, we identified specific chromosomal regions of the *Sphagnum* genome that correlate with diazotroph colonization. To provide a start to this analysis, the colonization effect size was used as a phenotype that was correlated to genome regions in a QTL analysis (Figure 14 and Figure 15). From this analysis, extreme high and low colonizing genotypes were chosen for further research. From Figures 14 and 15 we can see, based on colonization effect sizes, which *Sphagnum* genotypes have higher *Nostoc* colonization relative to those genotypes that do not get colonized well.

We hypothesize that this range in *Nostoc* colonization is due to the genetic variation underlying *Sphagnum fallax* colonization traits, and that the QTL analysis can identify specific genome regions mediating the *Sphagnum fallax* – cyanobacteria symbiosis. Figure 16 and 17 depict results from a QTL analysis using effect size measures of abundance acquired through two different estimates (nifH or POL primer pairs). Interestingly, both figures show similar peaks on chromosome 7 and 10 that is in linkage with *Nostoc* colonization estimated from both primer pairs. The blue and red peaks from both figure 16 and 17 are depicting 2 different statistical algorithms that were ran to measure the logarithm of odds ratio based on the phenotype of colonization effect size across the *Sphagnum* genome. This is the first attempt at explaining the variation in the genetic basis underlying this symbiosis.

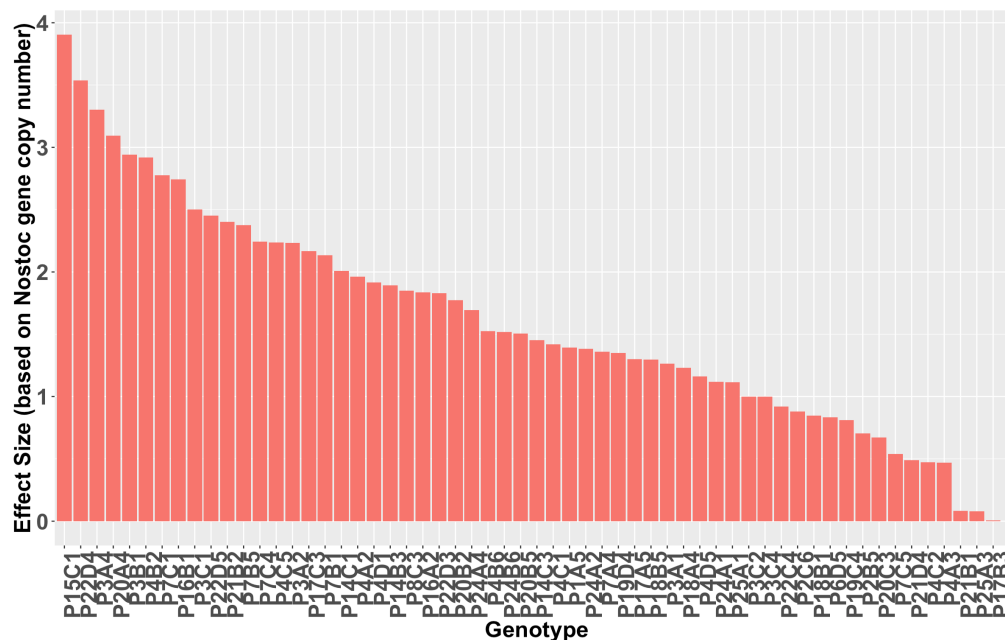


Figure 14- Effect size by each *Sphagnum* genotype while using *nifH* primer pair.

Effect size $((\text{treated-control}/\text{avg}(\text{treated-control})))$ plotted by corresponding *Sphagnum* genotype. Used to gain extreme genotypes (high/low (near 0) *Nostoc* colonizers) by *nifH* primer pair use.

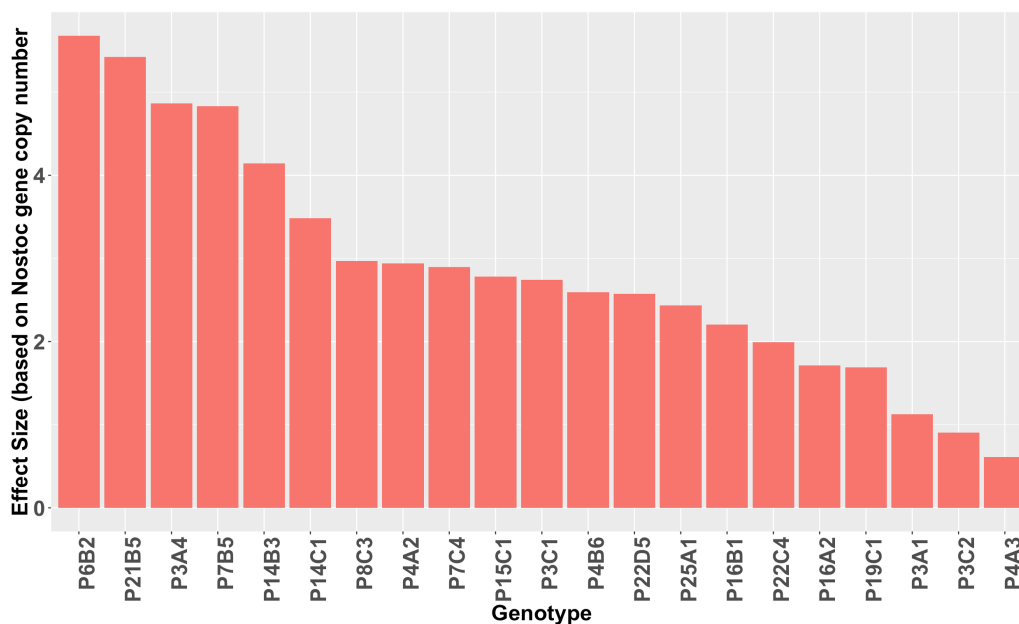


Figure 15- Effect size by each *Sphagnum* genotype while using *POL* primer pair.

Effect size $((\text{treated-control}/\text{avg}(\text{treated-control})))$ plotted by corresponding *Sphagnum* genotype. Used to gain extreme genotypes (high/low (near 0) *Nostoc* colonizers) by *POL* primer pair use.

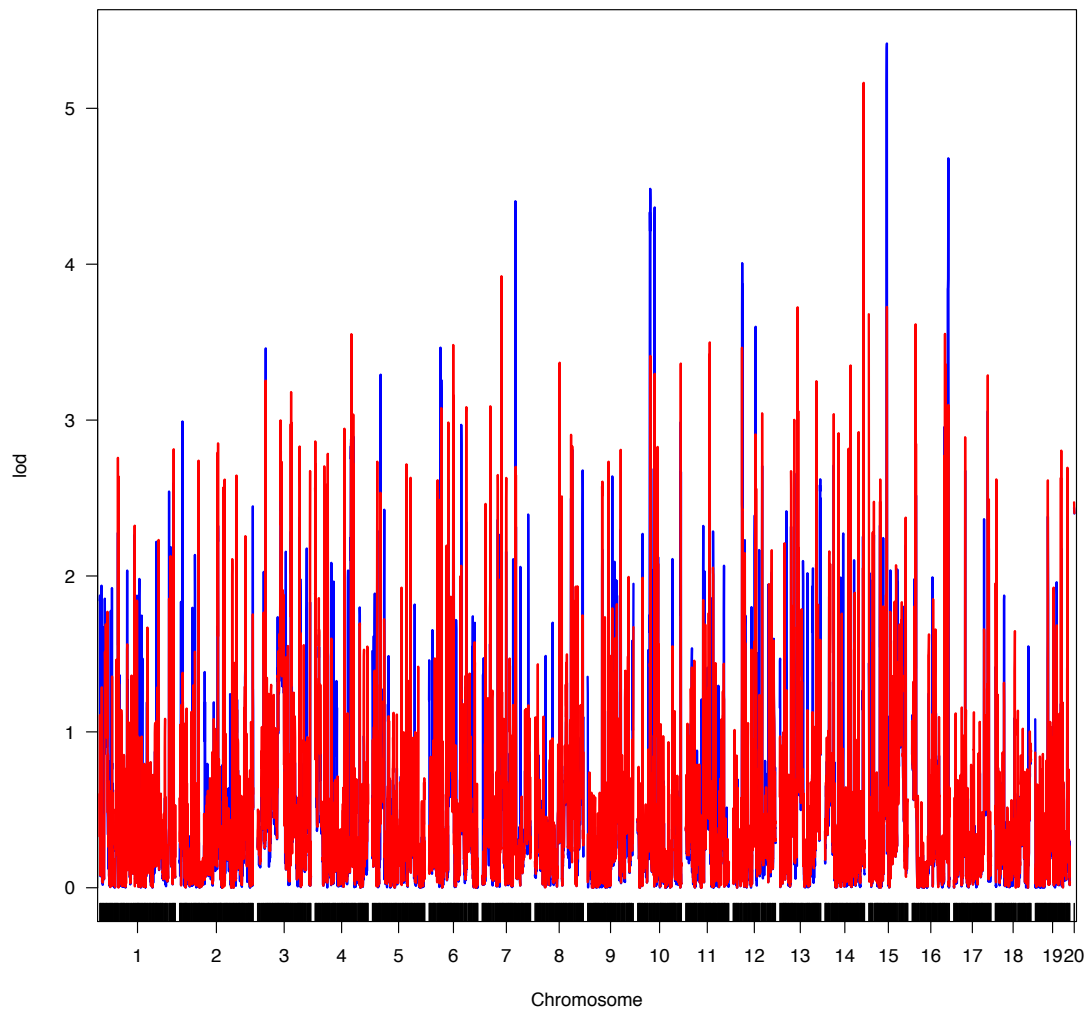


Figure 16- QTL plot of colonization effect size using *nifH* primer pair.

nifH primer pair colonization effect size phenotype data used to plot LOD scores across the *Sphagnum* genome.

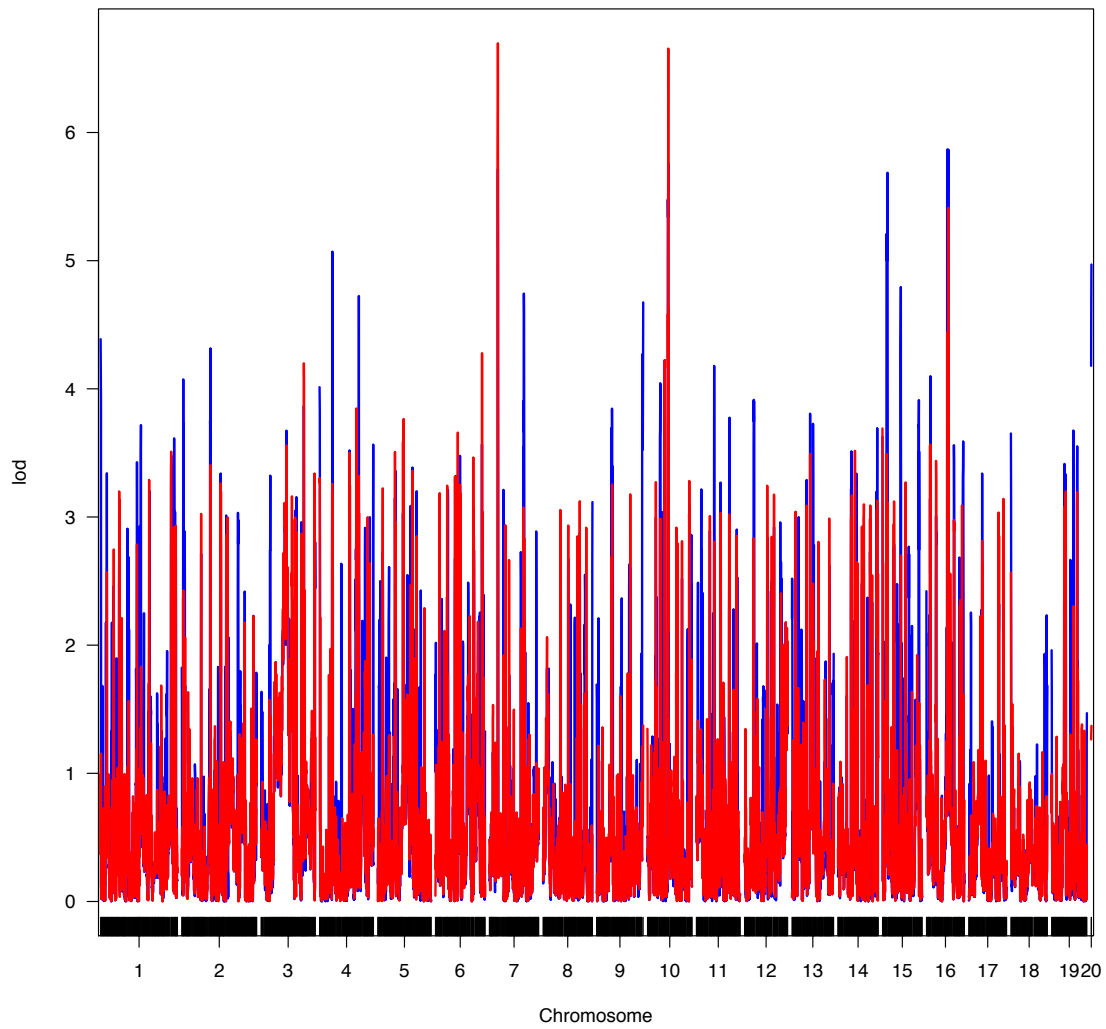


Figure 17- QTL plot of colonization effect size using POL primer pair.

POL primer pair colonization effect size phenotype data used to plot LOD scores across the *Sphagnum* genome.

4 | Discussion

In this study, we discovered that the *Sphagnum-Nostoc* symbiosis is truly mutualistic with both members benefitting from the interaction. However, the symbiosis is context dependent, where mutualism only occurs when the partners are interacting at a low pH environment. At relatively high pH the cyanobacteria seem to disproportionately benefit relative to the *Sphagnum* moss. Fitness costs and benefits of the interaction are dependent upon the pH at which they are grown. We also discovered through a supplemental experiment that both organisms are able to shift the aqueous pH to a more desirable environment while grown alone, however when grown in direct contact shift toward a neutral pH. Further, while at an optimal pH for mutualism to occur, we show that diazotroph colonization variation depends, in part, on *S. fallax* genetic variation through the identification of a putative QTL (quantitative trait locus). These results illustrate how environmental factors, such as pH, and plant host genetics can determine *Sphagnum-Nostoc* symbiosis outcomes.

It is believed that *Sphagnum* prefer acidic conditions as their fundamental niche, however I found that *Sphagnum* had no significant preference to the pH gradient when grown alone but interestingly shows a significant preference to acidic pH while in symbiosis with *Nostoc*. These results imply that the mutualism is allowing for a novel niche to be utilized, where each organism is able to persist at non-optimal conditions when in symbiosis together under acidic pH conditions. This suggests that while in symbiosis, the individual's niche can change or extend the range of necessary environmental conditions to allow the mutualism to endure.

Using *Sphagnum* as our model organism, we are able to apply molecular genetics to an ecologically relevant plant-microbe interaction. From this study we hypothesize, *Sphagnum* that are able to attain higher colonization of *Nostoc* have the potential to acquire more nitrogen and

thus greater productivity. This can lead to higher carbon assimilation and sequestration rates. This research will likely extend beyond to ecosystem level implications on carbon dynamics with further research conducted on the distribution of this symbiosis. For example, which species have greater effects on colonization and how much variation among populations is needed for substantial effects in C and N dynamics. Incorporating our findings into ongoing research with the SPRUCE project can be of great value since there are no genetic based studies in that project, especially those investigating plant-microbe interactions of the peatland ecosystem. This research is critical for understanding ecosystem functionality based on *Sphagnum* genetic variation and further research will allow us to estimate how much variation there is within this *Sphagnum* population. My study provides the foundation to add a potentially predictive genetic component to this interaction. For example, we could verify if our identified QTL region explains cyanobacteria abundance and diversity in the field, if there are QTL by temperature interactions, and do homologous genetic regions for this QTL exist in other mosses?

In the face of global change, this is an important study because we need to understand how ecosystems will thrive, maintain, or decline in regard to species interactions. *Sphagnum* genetic variation and pH are drivers of this ecologically relevant symbiosis; however, these factors alone will not allow us to fully understand or explain the C and N production in peatlands as climates warm. By utilizing this research, we can begin to make more precise estimates about C and N fixation rates based on this symbiosis and this will also allow us to draw more conclusions as to what warming will do to the symbiosis and the ecosystem as a whole.

Future plans are aimed at corroborating the QTL findings. First, we will use the extreme *Sphagnum* genotypes (high/low colonizers) that were found in the co-culture experiment and be able to replicate the experiment. Next, we will determine how much nitrogen is being fixed

based on the amount of *Nostoc* colonization. This can also be replicated with other *Sphagnum* species such as *S. fuscum*. This will give more understanding into how the moss are utilizing this nitrogen and to what extent. These additional studies will determine the functional strength of the QTL region in governing cyanobacteria colonization and nitrogen fixation, thereby enabling us to then test how the effects of warming may impact this critical symbiosis.

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Vita

Megan Patel graduated from the University of Tennessee Knoxville in 2016 with her Bachelor of Science degree in Biological Sciences with a concentration in Plant Biology. She worked in Dr. Joe William's laboratory as an undergraduate student in 2014 until she began an internship at Oak Ridge National Laboratory in 2015 under the mentorship of Lee Gunter. Megan gained many molecular, sterile, and greenhouse proficiencies. In 2018 she began her Master of Science program in Ecology and Evolutionary Biology with advisors Dr. Joseph Bailey (EEB) and Dr. David Weston (ORNL). Her thesis work focuses on a *Sphagnum fallax* population and the symbiosis that occurs with a nitrogen fixing cyanobacteria (diazotroph) to understand how a known diazotroph colonization can be driven by *Sphagnum* host genetic variation. She is active in community outreach efforts with the Roane county school systems STEM programs and also aided in UTK Darwin Day 2019 and 2020 campus outreach efforts.