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IDENTIFICATION OF *MASTIGOCLADUS LAMINOSUS* GENES ASSOCIATED WITH  
ENHANCED NITROGEN FIXATION PERFORMANCE

By

PATRICK ROSS HUTCHINS

B.S. Marine Science, Coastal Carolina University, Conway, SC, 2010  
M.S. Coastal Marine & Wetland Studies, Coastal Carolina University, Conway, SC, 2012

Thesis

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for the degree of

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Cellular, Molecular, and Microbial Biology

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Approved by:

Sandy Ross, Dean of the Graduate School  
Graduate School

Dr. Scott Miller, Committee Chair  
Division of Biological Science

Dr. Frank Rosenzweig, Committee Member  
Division of Biological Sciences

Dr. Cory Cleveland, Committee Member  
College of Forestry and Conservation

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## ABSTRACT

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Cellular, Molecular, and Microbial Biology

### IDENTIFICATION OF *MASTIGOCLADUS LAMINOSUS* GENES ASSOCIATED WITH ENHANCED NITROGEN FIXATION PERFORMANCE

Chairperson: Dr. Scott Miller

Understanding population variation for fitness-related traits is important for our comprehension of evolutionary adaptation and of how populations respond to environmental change. Here, I investigate variation in nitrogen fixation performance for an ecologically-variable population of the cyanobacterium *Mastigocladus laminosus* from White Creek, a nitrogen-limited, geothermally-influenced stream in Yellowstone NP. I next take a population genomics approach to identify candidate loci associated with superior performance. Variation among strains and temperature dependence of the nitrogen fixation process were the most important factors in a linear mixed effects model. Absolute and relative measures of genetic differentiation between strains from the upper quartile of nitrogen fixation performance and the other 75% of strains showed that only a small subset of loci were associated with superior nitrogen fixation. Most notably, the strains that fixed the most nitrogen contained a premature stop codon in a regulatory histidine kinase gene, but this allele was present at low frequency in other strains. Because this nonsense mutation eliminates many important functional sites in the protein, this allele is expected to be non-functional. Both the full-length and the putative null allele, as well as a third recombinant allele, were expressed during nitrogen step-down and in the presence of combined nitrogen. Future studies will investigate whether the nonsense mutation results in transcriptional rewiring that is favorable for nitrogen fixation.

Key Words: cyanobacteria, nitrogen fixation, thermophile, fitness, adaptation, genomics

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## Chapter 1

### **Abstract**

Understanding population variation for fitness-related traits is important for our comprehension of evolutionary adaptation and of how populations respond to environmental change. At a nitrogen-limited, geothermally-influenced stream in Yellowstone National Park, the cyanobacterium *Mastigocladus laminosus* fixes abundant nitrogen *in situ*, an important fitness-related trait in nitrogen-limited systems. While extensive work has been done to identify the genes required to perform nitrogen fixation, little is known about the amount or genetic basis of phenotypic variation in nitrogen fixation performance in natural populations. Here, I use standard acetylene reduction assays to quantify the extent of phenotypic variation for nitrogen fixation ability among 23 randomly-selected White Creek *M. laminosus* strains. Variation among strains and temperature dependence of the nitrogen fixation process were the most important factors in a linear mixed effects model. Genome-wide analysis of the assayed strains was next used to identify candidate genes that may contribute to enhanced nitrogen fixation performance. Absolute and relative measures of genetic differentiation between strains from the upper quartile of nitrogen fixation performance and the other 75% of strains showed that only a small subset of loci were associated with superior nitrogen fixation. Most notably, strains that fixed the most nitrogen contained a premature stop codon in a regulatory histidine kinase gene, but this allele was present at low frequency in other strains. Because this nonsense mutation eliminates many important functional sites in the protein, this allele is expected to be non-functional. Expression and functional assays are needed to identify the mechanism through which this putative null allele may confer enhanced nitrogen fixation performance.

### **Introduction**

Understanding population variation for fitness-related traits is important for our comprehension of evolutionary adaptation. As first pointed out by Darwin, heritable variation represents the raw material of evolution by natural selection. Forces that remove variation from a population, such as directional selection and genetic drift, are potentially counterbalanced by the input of mutations, gene flow, and balancing selection. Spatially-varying selection, for instance, is a form of balancing selection whereby spatial heterogeneity in the environment favors alternative genotypes (Hedrick, 2006). The extent of functional variation maintained in a population also has potential implications for both the resilience of ecosystem services in a changing environment (Hughes *et al.*, 1997; Luck *et al.*, 2003) as well as for how populations respond to temporal environmental change, because the rate at which beneficial mutations arise, and subsequently attain high frequencies, is slow in comparison to the speed at which populations can potentially adapt from standing genetic variation (Barrett, Schluter, 2008). Therefore, it is

37 vital that we understand the extent of standing phenotypic and genetic variation within  
38 populations and the mechanisms by which it is maintained.

39         At White Creek, a nitrogen-limited, geothermally-influenced stream in the Lower Geysers  
40 Basin of Yellowstone National Park, a population of the thermophilic, filamentous  
41 cyanobacterium, *Mastigocladus (Fischerella) laminosus*, exhibits tremendous ecological  
42 variation for temperature performance along a thermal gradient ranging from 39-54 °C mean  
43 annual temperature (Miller *et al.*, 2009). This strong temperature gradient exists in the presence  
44 of little apparent spatial variation in nutrient and light availability (Miller *et al.*, 2009). More than  
45 150 *M. laminosus* strains from five sampling locations spanning their natural range in White  
46 Creek have been archived and/or maintained in laboratory culture. *M. laminosus* strains from  
47 White Creek tend to grow better under laboratory conditions that mimic the mean temperatures  
48 from which they were originally collected, resulting in crossing reaction norms for temperature  
49 performance (Miller *et al.*, 2009). Although gene flow along White Creek is generally high  
50 throughout much of the genome, upstream and downstream strains of *M. laminosus* are  
51 genetically differentiated at specific genomic regions (Wall *et al.*, in press; Miller *et al.*,  
52 submitted). However, several other regions of the genome exhibit the signatures of balancing  
53 selection in the absence of obvious spatial structure. Questions remain regarding both the  
54 functional and the adaptive significance of this variation.

55         *M. laminosus* fixes abundant nitrogen *in situ* (Miller *et al.*, 2006), and it is expected that  
56 this is an important fitness-related trait in nitrogen-limited systems like White Creek. Biological  
57 nitrogen fixation is a globally significant biogeochemical process that many cyanobacteria  
58 perform. It is estimated that organisms that fix atmospheric nitrogen (diazotrophs) are  
59 responsible for more than half of global nitrogen fixation, in spite of increasing anthropogenic  
60 nitrogen fixation since the industrial era (Galloway *et al.*, 2004). Because cyanobacteria also  
61 perform oxygenic photosynthesis, they must perform two crucial metabolic processes that are at  
62 odds with one another. This is because the enzyme responsible for nitrogen fixation, nitrogenase,

63 contains a cofactor that is permanently deactivated by oxygen. In order to fix atmospheric  
64 nitrogen and perform oxygenic photosynthesis, cyanobacteria must separate these activities in  
65 either time or in space. Because photosynthetic oxygen production is light dependent, some  
66 cyanobacteria fix nitrogen under dark conditions, when their photosystems are naturally inactive  
67 (Berman-Frank *et al.*, 2001). An alternative strategy employed by *M. laminosus* and related  
68 cyanobacteria is to spatially separate these biochemically incompatible processes by means of  
69 specialized and terminally differentiated nitrogen-fixing cells called heterocysts.

70 Heterocysts, which are spaced at semi-regular intervals along filaments and typically  
71 account for ~5-10% of cells (Kumar *et al.*, 2010), have several important structural and functional  
72 differences from vegetative cells that enable nitrogen fixation to occur. Most importantly, the  
73 heterocyst creates a micro-oxic environment. The heterocyst's first defense against oxygen  
74 poisoning of nitrogenase is a physical barrier to oxygen diffusion in the form of an extracellular  
75 heterocyst envelope polysaccharide (HEP) and an underlying heterocyst glycolipid layer (HGL;  
76 Kumar *et al.*, 2010). Formation of the HEP layer is one of the earliest morphological changes  
77 during differentiation (Kumar *et al.*, 2010), and an intact HEP layer is required for heterocyst  
78 function in the presence of oxygen (Huang *et al.*, 2005; Wolk *et al.*, 1988), though it is generally  
79 believed that the HGL layer is the primary gas diffusion barrier (Currier *et al.*, 1977). Another  
80 measure taken during heterocyst development to enable nitrogenase activity under light  
81 conditions is the dismantling of the oxygen-producing photosystem (PS) II (Wolk *et al.*, 1994).  
82 An additional consequence of dismantling PSII is that the heterocyst is not able to generate  
83 reductant for carbon fixation (Wolk *et al.*, 1994). Consequently, fixed carbon in the form of  
84 sucrose is imported from adjacent vegetative cells to provide reducing power for nitrogen fixation  
85 (Kumar *et al.*, 2010). PS I, however, remains active, generating much of the ATP required for  
86 nitrogen fixation (Ernst *et al.*, 1983). Heterocysts also exhibit increased rates of respiration, the  
87 benefit of which is twofold: (1) intracellular oxygen is quickly consumed, which protects  
88 nitrogenase and (2) it provides a supplemental source of ATP that is used to power nitrogenase

89 (Wolk *et al.*, 1994). A return of combined nitrogen during the first 9-12 hours of heterocyst  
90 development can reverse the differentiation process , after which the cell is committed to  
91 differentiation (Yoon, Golden, 2001). Fixed nitrogen produced by heterocysts rapidly diffuses  
92 into adjacent vegetative cells (Popa *et al.*, 2007) via intracellular junctions (Mullineaux *et al.*,  
93 2008) and/or a continuous periplasm (Flores *et al.*, 2006).

94 Nitrogen fixation has a complex genetic basis in heterocystous cyanobacteria (Wolk,  
95 2000). In addition to nitrogen fixation (*nif*) genes that are common to most diazotrophs, genes  
96 involved in heterocyst differentiation are also required. Heterocyst differentiation has been  
97 extensively studied in the model cyanobacterium *Anabaena* PCC 7120 and is one of our best  
98 understood models of cell differentiation in bacteria (e.g., Kumar *et al.*, 2010). Nitrogen fixation  
99 is an energetically expensive process, and the heterocyst envelope is a significant investment,  
100 accounting for ~50% of cell dry weight (Dunn, Wolk, 1970); consequently, heterocysts are not  
101 produced when a preferred source of nitrogen is available in the environment. Within hours of  
102 nitrogen limitation, the master regulator of heterocyst differentiation, *hetR* (Buikema, Haselkorn,  
103 2001), is limited to the semi-regularly spaced 5-10% of cells destined to become heterocysts  
104 (Huang *et al.*, 2004). The number of genes estimated to be differentially regulated during  
105 heterocyst development is staggering, ranging from just over 1000 in *Anabaena* PCC 7120 (Ehira  
106 *et al.*, 2003) to just under 500 in *Nostoc punctiforme* (Campbell *et al.*, 2007). These include  
107 between 100-140 “*Fox*” genes that are required for nitrogen fixation in the presence of oxygen  
108 (Wolk, 2000). For instance, the development of a heterocyst that is functional in an oxic  
109 environment requires the coordinated expression of genes which remodel the cell surface to  
110 provide a passive gas diffusion barrier that limits the entry of oxygen (Nicolaisen *et al.*, 2009; see  
111 above). Nitrogen fixation (*nif*) genes are expressed late in development, roughly 24 hours after  
112 nitrogen deprivation in *Anabaena* PCC 7120 (Ehira *et al.*, 2003). There are at least 18 genes and  
113 two excised DNA elements arranged in two separate gene clusters controlled by 4 operons in the  
114 *Anabaena* PCC 7120 *nif* regulon (reviewed in Böhm, 1998).

115           While extensive work has been done to identify the genes required to develop a  
116 heterocyst and to perform nitrogen fixation, very little is known about either the amount or the  
117 genetic basis of phenotypic variation for this important biogeochemical process in natural  
118 populations. Here, I first address the extent of phenotypic variation for nitrogen fixation ability  
119 among 23 randomly-selected White Creek *M. laminosus* strains. To assess whether nitrogen  
120 fixation co-varies with divergent temperature-specific growth in upstream and downstream sub-  
121 populations of *M. laminosus*, nitrogen fixation was tested at both of the approximate temperature  
122 extremes of their natural range in White Creek. Because nitrogen fixation requires a significant  
123 amount of ATP, the provision of fuel by carbon fixation is likely an important co-occurring  
124 process in *M. laminosus* under nitrogen limitation (Kumar *et al.*, 2010). Thus, simultaneous  
125 measurements of nitrogen- and carbon-fixation were performed to investigate the expectation that  
126 these two crucial metabolic processes are positively correlated in *M. laminosus*.

127           I next build on existing genomic resources available for White Creek *M. laminosus*  
128 strains to take a population genomics approach to identify loci associated with superior  
129 performance for nitrogen fixation and its temperature dependence. Population genomics  
130 approaches are powerful tools that use genome-wide sampling of population genetic variation  
131 to detect candidate genes which potentially contribute to population differentiation or phenotypic  
132 variation, as evidenced, for example, by outlier levels of genetic differentiation (reviewed by  
133 Luikart *et al.*, 2003 and Storz 2005). Although population genomics approaches have  
134 transformed the study of adaptation and genetic disease in both model and non-model eukaryotic  
135 systems, these methods have only recently been applied to bacteria (e.g. Thomas *et al.*, 2012 and  
136 Epstein *et al.*, 2012). Previous genome-wide analysis of genetic differentiation of *M. laminosus*  
137 along the White Creek temperature gradient has demonstrated that only a small fraction of White  
138 Creek *M. laminosus* loci are highly differentiated between upstream (>50°C) and downstream  
139 (<50°C) sites (Wall *et al.*, submitted; Miller *et al.*, submitted). My general approach was to group

140 strains for which genome data were available into phenotypic classes based on nitrogen fixation  
141 performance and use these classes in analyses of genetic differentiation to identify candidate  
142 genes that may contribute to high metabolic performance under contrasting temperature regimes.  
143 This study provides new insights into the genetic basis of a globally important and biochemically  
144 complex metabolic process and on the influence of environment on the maintenance of diversity.

145

## 146 **Methods**

### 147 Nitrogen and carbon fixation assays

148         Axenic *M. laminosus* filaments were transferred to 125 mL Erlenmeyer flasks with 75  
149 mL of sterile D medium (Castenholz, 1988) and allowed to grow for at least two weeks. Once  
150 sufficient biomass accrued in D medium flasks, sub-samples were transferred to flasks with ND  
151 medium (D medium without combined nitrogen) to establish steady state growth in the absence of  
152 combined nitrogen, as in Miller *et al.* (2006) and Miller *et al.* (2009). White Creek *M. laminosus*  
153 strains were grown at the standard maintenance temperature of 50°C in ND medium at a light  
154 intensity of  $105 \pm 5 \mu\text{E m}^{-2} \text{s}^{-1}$  provided by cool white fluorescent bulbs. After two weeks, cultures  
155 were split into six sub-lines, with three each of these moved to 37°C and 55°C growth chambers,  
156 respectively. Sub-lines were maintained in each incubator in ND medium and with a 12/12 hr  
157 light/dark cycle ( $105 \pm 5 \mu\text{E m}^{-2} \text{s}^{-1}$  during the light cycle) for two weeks leading up to the assay.  
158 Sub-lines were transferred on days seven and twelve during this acclimation period to ensure that  
159 cells were in exponential growth phase on the day of the assay (14 days after cultures were split  
160 into sub-lines). For each strain, nitrogen fixation incubation assays were performed two separate  
161 times using independent starting cultures.

162         Sub-samples from each sub-line were homogenized using a tissue grinder and normalized  
163 to an  $\text{OD}_{750}$  of  $0.05 \pm 0.003$ . Cultures were homogenized such that large clumps of trichomes (i.e.  
164 chains of cells) were broken up, but long chains containing vegetative cells and heterocysts  
165 remained intact. Relative nitrogen fixation rates were estimated by the standard acetylene

166 reduction assay (ARA; Stewart *et al.*, 1967). Because the production of ethylene is proportional  
167 to the activity of the nitrogenase enzyme, “nitrogen fixation performance” will be used  
168 interchangeably with “normalized ethylene production” throughout this manuscript. Assays were  
169 carried out with 10 mL of ND medium in 20 mL crimp-sealed vials with a light and a dark  
170 replicate for each sub-line. Samples were incubated for four hours following the addition of 5  
171 mL of acetylene gas (generated by the addition of 5 g of calcium carbide to 100 mL of deionized  
172 water) at the beginning of the light cycle of the established light regime. Incubations were  
173 terminated by aspirating as much sample headspace as possible (~15 mL) from each incubation  
174 vial and injecting it into a pre-evacuated 5 mL crimp vial. Ethylene production was measured  
175 using flame-ionization detection gas chromatography (FID-GC) with a Shimadzu GC-2014.  
176 Ethylene production measurements were estimated using a standard curve, blank corrected  
177 against parallel incubation vials that contained only ND growth medium and normalized to an  
178 optical density of 0.050. Optical density was empirically determined to have a linear relationship  
179 with cell dry mass for *M. laminosus* samples (Pearson correlation,  $R^2 = 0.95$ ,  $p < 0.001$ ).  
180 Microscopic counts of heterocyst frequency were performed for one representative sub-line at  
181 each temperature treatment. This was done to ensure that any variability between strains in their  
182 ability to form heterocysts was taken into account during data analysis.

183 Concurrent estimations of carbon fixation by each sub-line were made using  $^{14}\text{C}$ -  
184 bicarbonate incorporation rates (see Miller et al. 1998). Briefly, incubations were initiated with  
185 the addition of 0.2  $\mu\text{Ci}$  of  $^{14}\text{C}$ -bicarbonate to 3 mL aliquots of each sub-line, carried out for one  
186 hour under the same light and temperature conditions as in the acetylene reduction assay above  
187 and then terminated with the addition of 200  $\mu\text{L}$  of formalin. To correct for non-biological  
188 uptake of radiolabeled carbon, formalin was added to a duplicate aliquot of one of the three sub-  
189 lines at each temperature treatment at the start of the incubation. The full 3 mL sample volume  
190 was filtered onto a 0.45  $\mu\text{m}$  GN-6 membrane filter (PALL Life Sciences), rinsed first with 3%  
191 HCl to remove unincorporated radioisotope, and then rinsed with deionized water. Filters were

192 then placed into 20 mL scintillation vials and allowed to ventilate in a fume hood for at least one  
193 hour before adding 1.5 mL of EcoLite scintillation fluid (ICN). Samples were then read by a  
194 Beckman LS6000SE scintillation counter. As with ethylene production, carbon fixation rates  
195 were normalized to an optical density of 0.050.

#### 196 Statistical Analysis

197 Because of the crossed experimental design and the heteroskedastic nature of the data,  
198 even after transformation, I generated a linear mixed effects model using the R “lme4” package  
199 (Bates *et al.*, 2014) to understand which factors explained the variation in observed nitrogen  
200 fixation performance. Fixed factors of the model were (1) normalized carbon fixation rate, (2)  
201 temperature treatment, (3) heterocyst frequency, and all possible interactions. The random effects  
202 structure was designed such that the model accounted for variation within incubations and among  
203 strains across the two temperature treatments. Other variables in the model were removed via  
204 backwards stepwise nested hypothesis testing using the F-test until the lowest Akaike information  
205 criterion score was obtained. A post-hoc pseudo-R<sup>2</sup> for linear mixed models (Nakagawa,  
206 Schielzeth, 2013) was used to approximate the fit of the model and estimate the amount of  
207 variation that could be explained by fixed factors and individual random effects.

#### 208 Identification of Candidate Genes

209 Results from the ARA’s were used to categorize *M. laminosus* strains into phenotypic  
210 classes based on normalized ethylene production within each temperature treatment (37 and 55 °C  
211 datasets) and overall pooled performance (pooled dataset). For each dataset, strains in the upper  
212 quartile of mean normalized ethylene production were binned as the “upper” phenotypic class and  
213 those below this benchmark categorized as the “lower” phenotypic class. Genomic data for *M.*  
214 *laminosus* strains used in the analysis were obtained previously (Miller *et al.*, submitted). Briefly,  
215 paired-end Illumina sequence data were obtained for 20 White Creek strains randomly-selected  
216 from the lab strain collection. Draft genomes were assembled *de novo* using Velvet (Zerbino,  
217 Birney, 2008). Contigs in these draft genomes were auto-annotated with the RAST server and



218 saved in GenBank format (Aziz *et al.*, 2008). For each genome, protein-coding genes (CDS)  
219 were extracted from the GenBank files with custom Perl scripts to create FASTA-formatted files  
220 of all CDS.

221 Sequential local BLASTn queries of a non-redundant database of CDS were used to build  
222 separate FASTA-formatted files of orthologous CDS corresponding to each locus for the two  
223 phenotypic classes described above. Only full-length CDS were included, and loci for which  
224 fewer than 10 sequences were available were excluded. Custom Perl scripts (Miller *et al.*,  
225 submitted) were then used to estimate genome-wide relative ( $F_{ST}$ ) and absolute ( $D_{XY}$ ) genetic  
226 differentiation of polymorphic loci between phenotypic classes. Though  $F_{ST}$  has historically been  
227 used to estimate the relative genetic variation between geographically distinct populations  
228 (Holsinger, Weir, 2009),  $F_{ST}$  may be applied to any pair of defined groups. Here, the groups of  
229 interest are based on phenotypic classes rather than geographic location. The resulting  
230 distributions of  $F_{ST}$  and  $D_{XY}$ , respectively, were taken as empirical null distributions for each  
231 dataset to avoid assumptions regarding demographic history used by model-based approaches for  
232 identifying candidate loci. Vetted outlier loci (top ~1% of the tail, 20 CDS) of both  $F_{ST}$  and  $D_{XY}$   
233 distributions were further explored by comparing them to available annotated orthologs in the  
234 NCBI database.

235

## 236 **Results & Discussion**

### 237 *Nitrogen Fixation Activity*

238 Strain means for normalized ethylene production values in the pooled dataset used to  
239 develop the model spanned a large range, from 0.35 PPM hr<sup>-1</sup> in WC434 to 12.36 PPM in  
240 WC245 (Table 1.1). There was very little variation in normalized ethylene production in dark  
241 treatments and these rates were, on average, 28% of normalized ethylene production in respective  
242 light conditions, which is consistent with other studies on various diazotrophs that report light-  
243 independent nitrogenase activities at less than half of those under saturating light (e.g. Liengen,

244 1999; Staal *et al.*, 2001; Fig. 1.1B). Only data from the light treatments were included in the  
245 model and in subsequent analyses.

246 The final linear mixed model used to estimate normalized ethylene production from the  
247 pooled dataset (Table 1.2) was a random slope model that included two fixed factors (heterocyst  
248 frequency and a carbon fixation by temperature interaction) and two random effects (incubation,  
249 and a strain by temperature interaction). The pseudo- $R^2$  ( $R^2_{(c)}$ ) for this model was 0.74 with fixed  
250 factors explaining 21% of the variation ( $R^2_{(m)}$ ), and random effects explaining the remaining 53%.  
251 Of the random effects, the strain of *M. laminosus* assayed accounted for 19% of the model  
252 variance, temperature accounted for 20% and incubation for 3% (the remaining 11% of variation  
253 is the residual for random effects). The strain, and thereby the genomic background, proved to be  
254 very influential in determining overall normalized ethylene production, accounting for more than  
255 one fourth of the total variation explained by the model.

256 Heterocyst frequencies were on average  $2.4 \pm 0.3\%$  (error based on 95% confidence  
257 interval). This is lower than the ~5-10% frequency that is typically reported for model heterocyst  
258 forming cyanobacteria but comparable to previous results obtained in the lab for *M. laminosus*  
259 under these conditions (unpublished data). The correlation between heterocyst frequency and  
260 normalized ethylene production was only significant in the 55 °C dataset (Pearson correlation,  $R$   
261 = 0.50,  $p < 0.01$ ), but was weakly positive in the 37 °C and pooled datasets ( $R = 0.17$  and 0.30,  
262 respectively). Mean strain-specific normalized carbon fixation rates for the pooled dataset ranged  
263 between 33 and 133  $\mu\text{g C L}^{-1} \text{ hr}^{-1}$  (data not shown). Normalized carbon fixation rates were  
264 generally higher in the 37 °C dataset than the 55 °C (averages of  $107 \pm 10$  and  $33 \pm 3 \mu\text{g C L}^{-1} \text{ hr}^{-1}$ ,  
265 respectively). There was a highly significantly positive correlation between normalized carbon  
266 fixation and normalized ethylene production in the 55 °C (Pearson correlation,  $R = 0.73$ ,  $p < 0.01$ )  
267 and pooled datasets (Pearson correlation,  $R = 0.59$ ,  $p < 0.01$ ). The relationship between  
268 normalized carbon fixation and normalized ethylene production in the 37 °C dataset was positive,  
269 but not significant (Pearson correlation,  $R = 0.50$ ,  $p > 0.05$ ). While the fixed factors described

270 above cumulatively explained a moderate amount of variation in the model (21%, Table 1.2),  
271 strain and temperature effects explained approximately twice as much model variation.

272 Normalized ethylene production varied widely across temperature treatments and strains  
273 (Fig. 1.1, Table 1.1). Out of the 23 strains assayed, 9 had reaction norm slopes that were  
274 significantly different from zero in the light treatments (Fig. 1.1A; t-test,  $p < 0.05$ ) and 8 in the  
275 dark treatments (Fig. 1.1B). Strains with non-zero slopes usually performed better at the lower  
276 temperature than at the higher temperature. This finding is corroborated by field  $^{15}\text{N}_2$  -uptake  
277 experiments with White Creek *M. laminosus* performed by Stewart (1970) and by acetylene  
278 reduction assays performed in the field at White Creek (Hutchins and Miller, unpublished). Just  
279 one strain (WC344) had higher average nitrogenase activity at 55 °C than at 37 °C. There was no  
280 correlation between the temperature at which each strain was isolated from White Creek and  
281 normalized ethylene production in either temperature-specific or pooled datasets (Pearson  
282 correlation,  $p > 0.05$ ; data not shown). The intrinsic temperature dependence of nitrogen fixation  
283 performance in the strains assayed here therefore does not appear to be tightly coupled to the  
284 location of strain origin along the thermal gradient. In the pooled dataset, strains in the upper  
285 phenotypic class were, not surprisingly, often also those that were in the upper class for  
286 temperature-specific normalized ethylene production (Table 1.1). The upper classes for 55 °C  
287 and the pooled dataset shared more common strains with each other than either did with those of  
288 the 37 °C group. Strains WC119, WC245, and WC439 were in the upper class for both of the  
289 temperature-specific and the pooled datasets.

#### 290 Genome-wide Analysis of Loci Associated with Nitrogen Fixation

291 Genomic data was available for five out of the six strains in the upper class (top quartile  
292 of pooled normalized ethylene production; WC119, WC1110, WC245, WC344, and WC439) and  
293 for 11 out of the 17 remaining (lower class) strains. The majority of the *M. laminosus* genome  
294 exhibited very little differentiation between phenotypic classes for all three datasets but contained  
295 distinct outliers in the tails of the distributions ( $F_{ST}$  and  $D_{XY}$  near zero; Fig. 1.2 and 1.3,

296 respectively). Candidate loci exhibited the greatest genetic differentiation between classes in the  
297 55 °C dataset. Nearly half of the candidate genes identified in the results did not have a homolog  
298 in the NCBI database with a known function (Fig. 1.4 and 1.5, Tables 1.3 – 1.8). However, for  
299 those that did have an identifiable function, the vast majority were involved with carbohydrate,  
300 amino acid, or inorganic ion transport/metabolism. The small peaks in the frequencies of  $F_{ST}$   
301 values centered on 0.30 – 0.35 for all three datasets are the result of the genetic clustering of a  
302 few strains from the lower class (WC1110, WC527, WC538, and WC441) for a sub-set of loci  
303 that are not associated with enhanced nitrogen fixation.

304 At 37 °C, the most represented cluster of orthologous groups (COG) category among  
305 candidates were those with unknown function or general prediction only (Fig. 1.4A and 1.5A,  
306 respectively). Those with identifiable functions were most commonly involved with inorganic or  
307 amino acid transport/metabolism or cell membrane biogenesis. However, there were several  
308 noteworthy candidate genes in the ~1% tail of outlier loci. One of the genes that appeared in tails  
309 of both the  $F_{ST}$  and  $D_{XY}$  distributions was candidate 19-42545. It is annotated as a diguanylate  
310 cyclase, an enzyme which is observed in diverse branches of the prokaryotic tree (Galperin,  
311 2004). Diguanylate cyclases catalyze the formation of 3'-5' cyclic diguanylic acid (c-di-GMP), a  
312 secondary messenger protein involved in numerous networks (Hengge, 2009) that leads to the  
313 biosynthesis of adhesins and exopolysaccharides associated with bacterial biofilm formation  
314 (Jenal, 2004). There are two nonsynonymous polymorphisms at this locus: all strains in the upper  
315 phenotypic class had a serine rather than an alanine at residue 34 and an aspartic acid instead of  
316 an asparagine at residue 42 (the allele fixed in the upper class was present at 42% frequency in  
317 the lower class). The highest  $F_{ST}$  value belonged to a potassium channel protein gene (candidate  
318 56-42545) orthologous to *alr0440* in *Anabaena* PCC 7120. This gene is upregulated during  
319 nitrogen step-down and heterocyst development (Ehira *et al.*, 2003), but differentiation between  
320 phenotypic classes was manifested by two synonymous polymorphisms, and its role in nitrogen  
321 fixation performance is not known (the allele fixed in the upper class was present in 40% of

322 strains in the lower class). Candidate 131-35450 is annotated as *hupW*, a protease that is involved  
323 in the maturation of the uptake hydrogenase (Wang *et al.*, 2012) and is upregulated during  
324 heterocyst development (Ehira *et al.*, 2003). The uptake hydrogenase recycles the hydrogen  
325 byproduct generated by nitrogenase, providing additional electrons that are used for nitrogen-  
326 reduction during fixation (Lindberg *et al.*, 2012). Inactivation of *hupW* results in a  
327 malfunctioning uptake hydrogenase and the evolution of excess hydrogen atoms in heterocysts  
328 (Lindberg *et al.*, 2012), thus decreasing the reducing power available to the heterocyst (Carrasco  
329 *et al.*, 2005). All of the upper class strains at 37 °C were characterized by a methionine at residue  
330 28, rather than an isoleucine, in the nickel binding site (the allele fixed in the upper class was  
331 present at 62% frequency in the lower class). Functional analyses of proteins from these  
332 candidate loci are needed to elucidate their effects on nitrogen fixation and fitness in *M.*  
333 *laminosus*.

334         Though the number of candidate genes encoding proteins with either unknown function  
335 or having only a general prediction was also high in the 55 °C dataset, a large proportion of the  
336 genes encoded proteins that are involved with carbohydrate transport and metabolism (Fig. 1.4B  
337 and 1.5B for  $F_{ST}$  and  $D_{XY}$ , respectively). The locus with the greatest  $F_{ST}$  value was candidate 28-  
338 39736, an adenylylsulfate (APS) kinase. These phosphotransferases catalyze the second reaction  
339 of the conversion of inorganic sulfate to 3'-phosphoadenosine 5'-phosphosulfate as part of  
340 assimilatory sulfur metabolisms (Renosto *et al.*, 1984). A single nonsynonymous polymorphism  
341 between the two classes was present at nucleotide 241, resulting in an aspartic acid in the upper  
342 class while the majority of lower class strains contain an asparagine at this position (the allele  
343 fixed in the upper class was present at 17% frequency in the lower class). Candidate 1-33964  
344 encodes the hopene-associated glycosyltransferase, *hpnB*. Glycosyltransferases which contain  
345 family 2 domains, as is the case with *hpnB*, are generally responsible for transferring nucleotide-  
346 diphosphate sugars to polysaccharide and lipid substrates (Perzl *et al.*, 1998). *hpnB* (alr0776) is  
347 one of several genes related to heterocyst development that is upregulated by NaCl in the

348 cyanobacterium, *Anabaena* sp. PCC 7120 (Imashimizu *et al.*, 2005). All of the strains in the  
349 upper phenotypic class possessed a nonsynonymous polymorphism that translates to an alanine  
350 instead of a glycine at residue 271 (the allele fixed in the upper class was present in 27% of  
351 strains in the lower class). The third  $F_{ST}$  outlier, candidate 49-34361, is annotated as a cation-  
352 transporting ATPase and shows weak homology to all3375 in the *Anabaena* PCC 7120 genome  
353 (Kaneko *et al.*, 2001). A nonsynonymous polymorphism between the phenotypic classes resulted  
354 in a proline in the upper class and a leucine in the lower class at amino acid 172 (the allele fixed  
355 in the upper class was present at 33% frequency in the lower class). Candidate 65-42545 encodes  
356 the third subunit of cytochrome oxidase that is most similar to the homologous gene located in the  
357 *coxBACI* operon in *Anabaena* PCC 7120. While this gene is mildly upregulated during  
358 heterocyst development, it does not appear to be the primary cytochrome oxidase responsible for  
359 enhanced respiratory activity within the heterocyst (Jones, Haselkorn, 2002). Furthermore, the  
360 interaction between cytochrome-c and cytochrome oxidase occurs on subunits I and II, whereas  
361 the third subunit is not involved catalytically (Witt *et al.*, 1998). The differentiation between  
362 classes manifested as a synonymous adenine instead of guanine at nucleotide 435 in the gene (the  
363 allele fixed in the upper class was present in 36% of strains in the lower class). Still, consumption  
364 of intracellular oxygen is crucial for heterocysts, and genetic changes in this gene may potentially  
365 contribute to variation in nitrogen fixation among strains of *M. laminosus*. Candidate 93-42545 is  
366 homologous to alr4726 in the *Anabaena* PCC 7120 genome, which encodes a protein that has  
367 been identified as belonging to the zinc uptake regulator family of sensory kinases. Two  
368 synonymous polymorphisms are observed between phenotypic classes: in strains from the upper  
369 class a cytosine is present instead of a thymine at nucleotide 252 and thymine rather than a  
370 cytosine at 261 (the allele fixed in the upper class was present at 36% frequency in the lower  
371 class).

372           Though in many cases, the polymorphisms that distinguish phenotypic classes at the  
373 above candidate loci are synonymous, it does not necessarily mean that these loci are

374 unimportant. While selection may act on codon usage, an alternative possibility is that the actual  
375 target of selection is in non-coding DNA that is physically linked to the candidate locus. The  
376 analysis screened protein coding regions of the *M. laminosus* genome, but polymorphisms in non-  
377 coding regulatory regions that are adjacent to genes may potentially influence nitrogen fixation  
378 performance. A sliding window approach along genome contigs could be used to find adjacent,  
379 physically linked non-coding regions of the *M. laminosus* genome that may also be differentiated  
380 between phenotypic classes and which may be the true target of selection.

381         Within the pooled dataset, the most common functionally identifiable COGs among the  
382  $F_{ST}$  candidates were carbohydrate transport/metabolism, amino acid transport/metabolism, and  
383 signal transduction proteins. A histidine kinase gene, candidate 167-28586, was an outlier in both  
384 the  $F_{ST}$  and  $D_{XY}$  distributions and had the highest values for each respective metric of any gene  
385 for the pooled dataset. Unlike other candidates identified thus far, there appear to be three  
386 segregating alleles at this locus: an allele with a nonsense mutation that eliminates 150  
387 nucleotides at the 3' end of the gene (fixed in upper class, 36% frequency in lower class); a full-  
388 copy allele that differs from the above allele at 39 nucleotide positions, one of which includes the  
389 site of the alternative nonsense polymorphism; and an apparently rare ( $N = 1$  in our sample)  
390 recombinant allele that is identical to the latter at the 5' end and to the former at the 3' end and  
391 therefore contains the nonsense mutation (see 'A Histidine Kinase Candidate' below for more  
392 discussion of candidate 167-28586). Candidate 20-24813 is annotated as an enzyme in the  
393 cytochrome P450 family. P450s are heme-thiolate proteins that oxidize and degrade a diverse  
394 array of substrates and have equally diverse structures throughout all three domains of life  
395 (Werck-Reichhart, Feyereisen, 2000). Strains in the upper phenotypic class have a synonymous  
396 polymorphism at nucleotide 1317 in the form of a thymine (27% frequency in lower class), rather  
397 than a cytosine, as is the case with most strains in the lower class. Candidate 29-33117 encodes  
398 the cytochrome-c550 component of PS II. Cytochrome-c-550 is a membrane bound component  
399 of the cyanobacterial PS II oxygen-evolving complex (OEC; responsible for the water-splitting

400 reaction that produces oxygen and provides reducing power for carbon fixation) and is  
401 responsible for stabilizing chlorine and calcium-binding to the complex (Roncel *et al.*, 2012).  
402 Differentiation between the phenotypic classes was characterized by a single synonymous  
403 polymorphism: cytosine in the upper class and thymine in the lower class at nucleotide 471 (the  
404 allele fixed in the upper class was present at 50% frequency in the lower class). Candidate 10-  
405 32834 was in the upper 1% of both  $F_{ST}$  and  $D_{XY}$  distributions and is annotated as the nickel-  
406 binding alpha subunit of urease, an enzyme that catalyzes the hydrolysis of urea into ammonia  
407 and carbon dioxide (Holm, Sander, 1997). There were four nucleotide polymorphisms in the  
408 upper class, two of which are adjacent and are nonsynonymous. Strains in the upper class have  
409 an alanine at residue 555 (55% frequency in lower class) while some strains in the lower class  
410 have an allele with an asparagine at this location. The polymorphism described here does not  
411 occur in either a metal binding site or the active site of the protein, though it is possible that it  
412 confers a structural modification. Urease is directly involved in assimilatory nitrogen metabolism  
413 and the recycling of urea generated by cell metabolism. Chemical analysis of water samples  
414 taken along White Creek does suggest that there are occasional pulses of dissolved organic  
415 nitrogen in the system (Hutchins, unpublished). Urease's involvement in nitrogen metabolism  
416 and the differentiation in the alpha subunit gene between phenotypic classes presented here make  
417 this locus an interesting prospect for future investigations.

#### 418 *A Histidine Kinase Candidate*

419 The 167-28586 locus, which appears as a candidate in the pooled dataset and putatively  
420 encodes a histidine kinase, is particularly noteworthy. In addition to being an extreme outlier by  
421 both metrics of genetic differentiation, 167-28586 also exhibits the molecular evolutionary  
422 signatures of long-term balancing selection (Miller *et al.*, submitted). These include an extremely  
423 positively skewed value of Tajima's  $D$  and an excess of polymorphism in the White Creek  
424 population. Histidine kinases (HKs) are involved in two-component signal transduction systems  
425 (TCSs), the principal means by which bacteria sense and respond to environmental changes (Gao,



426 Stock, 2009; Wuichet *et al.*, 2010). Prototypical TCSs involve two distinct proteins. A histidine  
427 kinase (HK), which usually has a sensory domain that interacts with the intra- or extracellular  
428 environment, serves as the input component of the system. The HK then transfers phosphoryl  
429 groups to a cognate response regulator (RR) to effect a change in gene expression or, sometimes,  
430 protein activity (Galperin, 2010). Once stimulated, a well-conserved ATP binding domain at the  
431 C-terminal end of the HK catalyzes the autophosphorylation of a conserved histidine residue.  
432 The phosphorylated HK then transfers the His-bound phosphoryl group to an asparagine residue  
433 in a highly-conserved receiver domain on the RR.

434         167-28586 exhibits ~50% amino acid identity with three histidine kinases in the  
435 *Anabaena* PCC 7120 genome (alr1551, alr2739, and alr4882). For several reasons, alr4882  
436 appears to be the ortholog in the *Anabaena* PCC 7120 genome. 67-28586 and alr4882 share the  
437 same length and domain structure (both lack a sensory domain) as alr4882, which is not the case  
438 with other *Anabaena* homologs. Also, local gene order in the region is conserved: both HK167-  
439 28586 and alr4882 are downstream of a putatively orthologous annotated gene encoding a protein  
440 with a FIST sensory domain that likely serves as the sensory component of this network (alr4881  
441 and the corresponding *M. laminosus* gene are ~56% identical at the amino acid level). While  
442 FIST domains are phylogenetically widespread, they are biochemically uncharacterized, though  
443 they are predicted to bind small molecules (Borziak, Zhulin, 2007).

444         It is likely that the loss of more than half of the ATP-binding pocket would render  
445 HK167-28586 nonfunctional, even if it were expressed. I propose that this would result in a  
446 transcriptional “rewiring” that is somehow favorable with respect to nitrogen fixation. Loss of  
447 function mutations that alter regulatory networks may be a common mechanism of bacterial  
448 adaptation to environmental change (Hottes *et al.*, 2013). “Gain-of-function” mutations require  
449 very specific alterations to genes and are rare compared to “loss-of-function” mutations, which  
450 can be explored rapidly in a large evolving population (Hottes *et al.*, 2013). However, in order  
451 for HK167-28586 to contribute to the observed differences in performance between phenotypic

452 classes, I expect that the full copy of the allele must, at the very least, be expressed during either  
453 nitrogen step-down and/or steady-state growth under nitrogen-limitation (see Chapter 2).

454 Interestingly, HK167-28586 is located just upstream of what appears to be an  
455 orthologous gene to *alr0677* in the *Anabaena* PCC 7120 genome. This gene exhibits homology  
456 with a site-specific recombinase, XisC, which is required for the excision of ~10.5 kilobases from  
457 the *hupL* gene in *Anabaena* PCC 7120, which encodes the large subunit of the uptake  
458 hydrogenase (Carrasco *et al.*, 2005). Excision of the *hupL* element is necessary to produce a  
459 functional heterocyst-specific [NiFe] uptake hydrogenase, which catalyzes the consumption of  
460 hydrogen that is produced as a byproduct of nitrogen fixation (Tamagnini *et al.*, 2002). Two  
461 other site-specific recombinases are also required for nitrogen fixation in *Anabaena* PCC 7120  
462 (Böhm, 1998). In order to produce a functional nitrogenase enzyme, two inserted elements in the  
463 *nif* operon must be removed: a 55 kilobase element from *fdxN*, which encodes a ferredoxin-like  
464 protein, and an 11 kilobase element from *nifD*, which encodes the alpha subunit of the  
465 nitrogenase MoFe protein. These elements contain genes encoding the site-specific recombinases  
466 required for their own excision: *xisF* and *xisA*, respectively. Though the proximity of Hk167-  
467 28586 to a putative site-specific recombinase is intriguing, at this time it is not possible to say  
468 what, if any, relationship exists between the two genes and nitrogen fixation performance in *M.*  
469 *laminosus*.

470

## 471 **Conclusion**

472 Differences among strains explained a considerable portion of the variation in nitrogen  
473 fixation in a mixed effects model. The comparatively low number of loci that were strongly  
474 associated with phenotypic variation among strains in nitrogen fixation performance suggests that  
475 dissecting the contributions of these genetic factors to variation in this complex trait may be  
476 tractable. However, the signatures of selection that we observe in our genome data may be the  
477 product of natural selection acting on subtle phenotypic differences that may be difficult to

478 resolve with laboratory experiments. Consequently, it may be challenging to quantify the impact  
479 of a locus (i.e., its effect size) on the phenotypic variation for a quantitative trait without large  
480 sample sizes. For example, although a positive relationship is estimated between ethylene  
481 production and the presence of the premature stop codon in HK167-28586 (Fig. 1.6), the model is  
482 not significant for this low sample size (Nagelkerke  $R^2 = 0.14$ ,  $p = 0.27$ ; Nagelkerke, 1991). A  
483 much larger sample will be required to obtain an accurate estimate of the contribution of this  
484 locus to variation in nitrogen fixation. However, the first step in determining how genetic  
485 variation at this locus may act to enhance nitrogen fixation performance is to identify its pattern  
486 of expression with respect to nitrogen limitation.

**Table 1.1** Strain means and errors (95% confidence) for normalized ethylene production and summary statistics for all strain means. Underlined values are those which were in the top quartile of their respective columns.

WC Strain	Normalized Ethylene Production (PPM hr <sup>-1</sup> )		
	37°C	55°C	Pooled
111	4.92 ±0.60	4.09 ±0.60	4.51 ±0.42
112	4.99 ±0.84	0.41 ±0.03	2.70 ±0.79
114	<u>12.36 ±1.30</u>	2.06 ±0.43	<u>7.21 ±1.66</u>
116	4.78 ±1.13	2.83 ±0.82	3.80 ±0.72
119	<u>7.18 ±0.75</u>	<u>5.49 ±1.32</u>	<u>6.33 ±0.76</u>
1110	<u>6.35 ±1.54</u>	1.80 ±0.45	4.08 ±1.02
213	3.49 ±0.67	2.57 ±1.09	3.07 ±0.60
217	1.35 ±0.22	1.90 ±0.41	1.62 ±0.24
245	<u>8.54 ±0.54</u>	<u>6.71 ±2.37</u>	<u>7.63 ±1.19</u>
246	5.68 ±0.51	2.17 ±1.26	4.27 ±0.78
249	1.94 ±0.23	1.81 ±0.41	1.88 ±0.23
326	<u>8.07 ±1.54</u>	0.68 ±0.33	4.04 ±1.33
344	4.91 ±1.34	<u>9.25 ±1.29</u>	<u>7.08 ±1.09</u>
434	0.30 ±0.04	0.41 ±0.06	0.35 ±0.04
438	5.71 ±0.41	<u>5.49 ±1.00</u>	<u>5.60 ±0.52</u>
439	<u>8.70 ±1.22</u>	<u>5.05 ±0.98</u>	<u>6.88 ±0.92</u>
441	5.58 ±1.25	4.36 ±0.81	5.03 ±0.76
442	4.03 ±0.95	2.47 ±0.68	3.25 ±0.60
527	4.78 ±0.64	2.37 ±0.55	3.58 ±0.54
538	4.79 ±0.50	3.06 ±0.68	3.92 ±0.48
542	5.52 ±0.44	<u>5.58 ±0.90</u>	5.55 ±0.45
558	4.56 ±0.45	2.67 ±0.53	3.61 ±0.43
559	2.91 ±0.47	2.34 ±1.14	2.62 ±0.59
Mean	5.28	3.29	4.29
Minimum	0.30	0.41	0.35
Lower Quartile	4.30	1.98	3.16
Median	4.92	2.57	4.04
Upper Quartile	6.03	4.71	5.57
Maximum	12.36	9.25	7.63

**Table 1.2** Mixed effects model summary using R's lme4 package syntax.  $R^2_{(m)}$  is the model variation explained by fixed factors and  $R^2_{(c)}$  is the total variation explained by the model.

Model	AIC	BIC	Log Likelihood	$R^2_{(m)}$	$R^2_{(c)}$
$ET \sim 1 + H + C \times T + (1 I) + (1+T S)$	3525.4	3556.7	-1752.7	0.21	0.74

Variables	Definition
C	$^{14}\text{C}$ -bicarbonate incorporation rate ( $\mu\text{g C hr}^{-1}$ )
ET	Normalized ethylene production ( $\text{PPM hr}^{-1}$ )
H	Heterocyst frequency (heterocysts per cell counted)
S	Nominal <i>M. laminosus</i> strain ID
T	Temperature treatment ( $^{\circ}\text{C}$ )

489

**Table 1.3** Candidate genes associated with variation in nitrogen fixation performance at 37 °C, the relative genetic differentiation between phenotypic classes ( $F_{ST}$ ), and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

Gene	$F_{st}$	Annotation
56-42545	0.4836	Potassium channel protein
59-39736	0.4563	Ribosomal protein S12 methylthiotransferase
124-17867	0.4258	Hypothetical Protein
223-48944	0.3992	Cobalt transport protein component
<b>19-42545</b>	<b>0.3966</b>	<b>Diguanylate cyclase</b>
131-35450	0.3961	Hydrogenase maturation protease hupW
227-48944	0.3886	Hypothetical Protein
44-39685	0.3750	HSP htpX
41-4197	0.3750	Hypothetical Protein
112-40954	0.3684	Hypothetical Protein
24-24749	0.3649	Hypothetical Protein
33-4197	0.3633	Hypothetical Protein
13-30518	0.3633	GCN5 family acetyltransferase
<b>31-39736</b>	<b>0.3584</b>	<b>Hypothetical Protein</b>
19-24813	0.3514	Hypothetical Protein
237-48944	0.3503	Macrolide ABC transporter/ATP-binding protein
38-47543	0.3468	Hypothetical Protein
<b>7-45</b>	<b>0.3379</b>	<b>Hypothetical Protein</b>
1-30518	0.3333	Group 1 glycosyl transferase
23-24749	0.3333	Hypothetical Protein

490

**Table 1.4** Candidate genes associated with variation in nitrogen fixation performance at 37 °C, the absolute genetic differentiation between phenotypic classes ( $D_{XY}$ ), and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

Gene	$D_{xy}$	Annotation
33-9029	0.0057	Hypothetical protein
<b>19-42545</b>	<b>0.0035</b>	<b>Diguanylate cyclase</b>
11-43020	0.0032	Hypothetical protein
12-14867	0.0031	Hypothetical protein
124-17867	0.0026	SCP-like extracellular protein
20-51983	0.0020	Hypothetical protein
<b>31-39736</b>	<b>0.0018</b>	<b>Hypothetical protein</b>
17-20539	0.0018	Hypothetical protein
44-43317	0.0018	tRNA(Ile)-lysidine synthase
118-37089	0.0018	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
45-9675	0.0017	Phosphate ABC transporter substrate-binding protein
243-48944	0.0016	Putative Anti-Sigma regulatory factor (Ser/Thr kinase)
68-28680	0.0016	Nitrate ABC transporter, inner membrane subunit
309-48944	0.0015	Hypothetical protein
128-40954	0.0015	Nucleotidyl transferase
44-29888	0.0014	Hypothetical protein
<b>7-45</b>	<b>0.0014</b>	<b>Hypothetical protein</b>
54-57682	0.0013	Hypothetical protein
69-28680	0.0013	Amino acid ABC transporter substrate-binding protein
228-48944	0.0012	Ferritin, Dps family protein

**Table 1.5** Candidate genes associated with variation in nitrogen fixation performance at 55 °C, the relative genetic differentiation between phenotypic classes ( $F_{ST}$ ), and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

Gene	$F_{st}$	Annotation
28-39736	0.6954	Adenylyl-sulfate kinase
1-33964	0.5698	Hopene-associated glycosyltransferase HpnB
49-34361	0.5273	Cation-transporting ATPase
65-42545	0.5176	Cytochrome-c oxidase subunit 3
93-42545	0.5150	Membrane-anchored histidine kinase
44-39378	0.4833	Hypothetical Protein
15-39736	0.4625	Teichoic acid-transporting ATPase/ABC transporter
48-34361	0.4611	Cation-transporting ATPase
35-24749	0.4600	Cyclic nucleotide binding
72-4197	0.4563	RNP-1 like binding protein
55-20539	0.4526	Hypothetical Protein
22-24813	0.4328	Hypothetical Protein
29-33117	0.4278	Cytochrome-c 550 psbV
69-4197	0.4172	Hypothetical Protein
<b>7-17867</b>	<b>0.4103</b>	<b>MFS transporter</b>
4-65273	0.4103	S-adenosylmethionine synthetase
59-13348	0.4082	ArsR family transcriptional regulator
12-39736	0.4074	Hypothetical Protein
<b>77-48944</b>	<b>0.4045</b>	<b>ABC transporter</b>
29-2411	0.4028	FAD dependent oxidoreductase

491

**Table 1.6** Candidate genes associated with variation in nitrogen fixation performance at 55 °C, the absolute genetic differentiation between phenotypic classes ( $D_{XY}$ ), and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

Gene	$D_{xy}$	Annotation
<b>77-48944</b>	<b>0.0081</b>	<b>ABC transporter</b>
10-32834	0.0053	Urease alpha subunit
31-20539	0.0039	Glycosyl transferase family 2
62-37089	0.0038	Chlorophyll A-B binding protein
93-17867	0.0036	Hypothetical protein
19-42545	0.0035	Hypothetical protein
12-17867	0.0033	ABC-type nitrate/sulfonate/bicarbonate transport system, ATPase
80-29888	0.0031	ABC-2 type transporter
92-17867	0.0029	Exodeoxyribonuclease VII small subunit
118-40954	0.0025	Hypothetical protein
102-32982	0.0025	Hydrogenase expression/formation protein HypD
34-39685	0.0024	Glycosyl transferase family 2
76-17867	0.0023	Cobalt transport protein
132-48944	0.0022	Putative ABC-type transport system, permease component
127-48944	0.0021	Basic membrane lipoprotein
84-29888	0.0021	Oxidoreductase domain protein
23-29888	0.0007	FHA domain containing protein
81-29888	0.0020	Teichoic-acid-transporting ATPase
<b>7-17867</b>	<b>0.0020</b>	<b>MFS transporter</b>
20-51983	0.0020	Hypothetical protein

**Table 1.7** Candidate genes associated with variation in pooled nitrogen fixation performance, the relative genetic differentiation ( $F_{ST}$ ) between phenotypic classes, and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

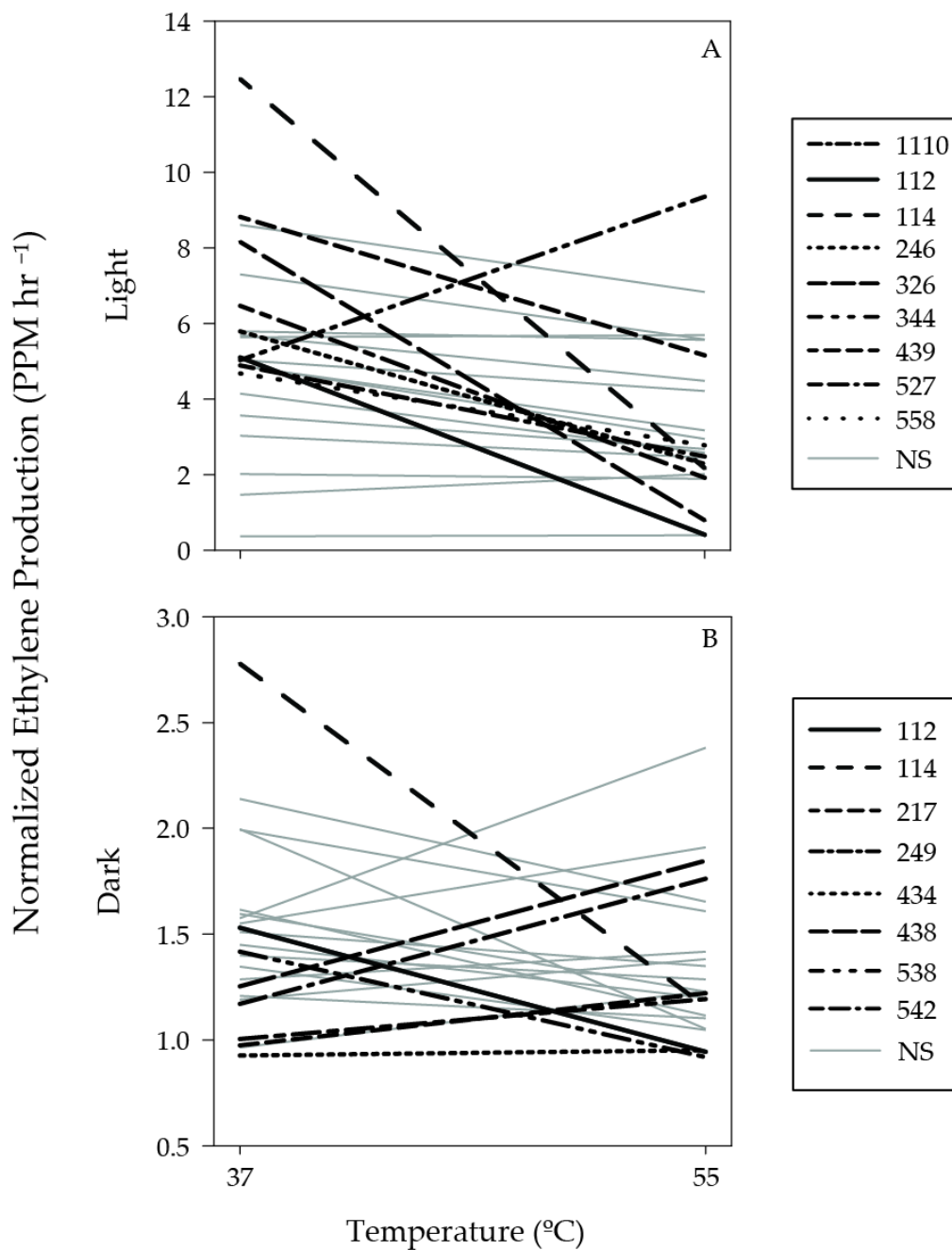
Gene	$F_{st}$	Annotation
<b>167-28586</b>	<b>0.5397</b>	<b>Histidine Kinase</b>
20-24813	0.5313	Cytochrome P450
<b>59-17867</b>	<b>0.5000</b>	<b>Hypothetical Protein</b>
15-39736	0.4813	Teichoic-acid-transporting ATPase/ABC transporter
72-4197	0.4563	RNP-1-like binding protein
<b>77-48944</b>	<b>0.4378</b>	<b>ABC transporter</b>
7-17867	0.4264	MFS transporter
22-24813	0.4179	Hypothetical Protein
29-33117	0.4175	Cytochrome-c550
12-51983	0.4141	Unknown
108-24813	0.4138	Cyclic nucleotide-binding protein
59-13348	0.4082	ArsR-family transcriptional regulator
12-39736	0.4074	Hypothetical Protein
57-16960	0.4057	Hypothetical Protein
<b>10-32834</b>	<b>0.4050</b>	<b>Urease alpha subunit</b>
29-2411	0.4028	FAD dependent oxidoreductase
66-15735	0.4000	Hypothetical Protein
<b>62-37089</b>	<b>0.4000</b>	<b>Chlorophyll a-b binding protein</b>
223-48944	0.3992	Cobalt transport protein component CbiN
<b>19-42545</b>	<b>0.3966</b>	<b>Unknown</b>

492

**Table 1.8** Candidate genes associated with variation in pooled nitrogen fixation performance, the absolute genetic differentiation between phenotypic classes ( $D_{XY}$ ), and corresponding annotations of homologous genes in the NCBI database. Emboldened rows are genes that are both  $F_{ST}$  and  $D_{XY}$  outliers.

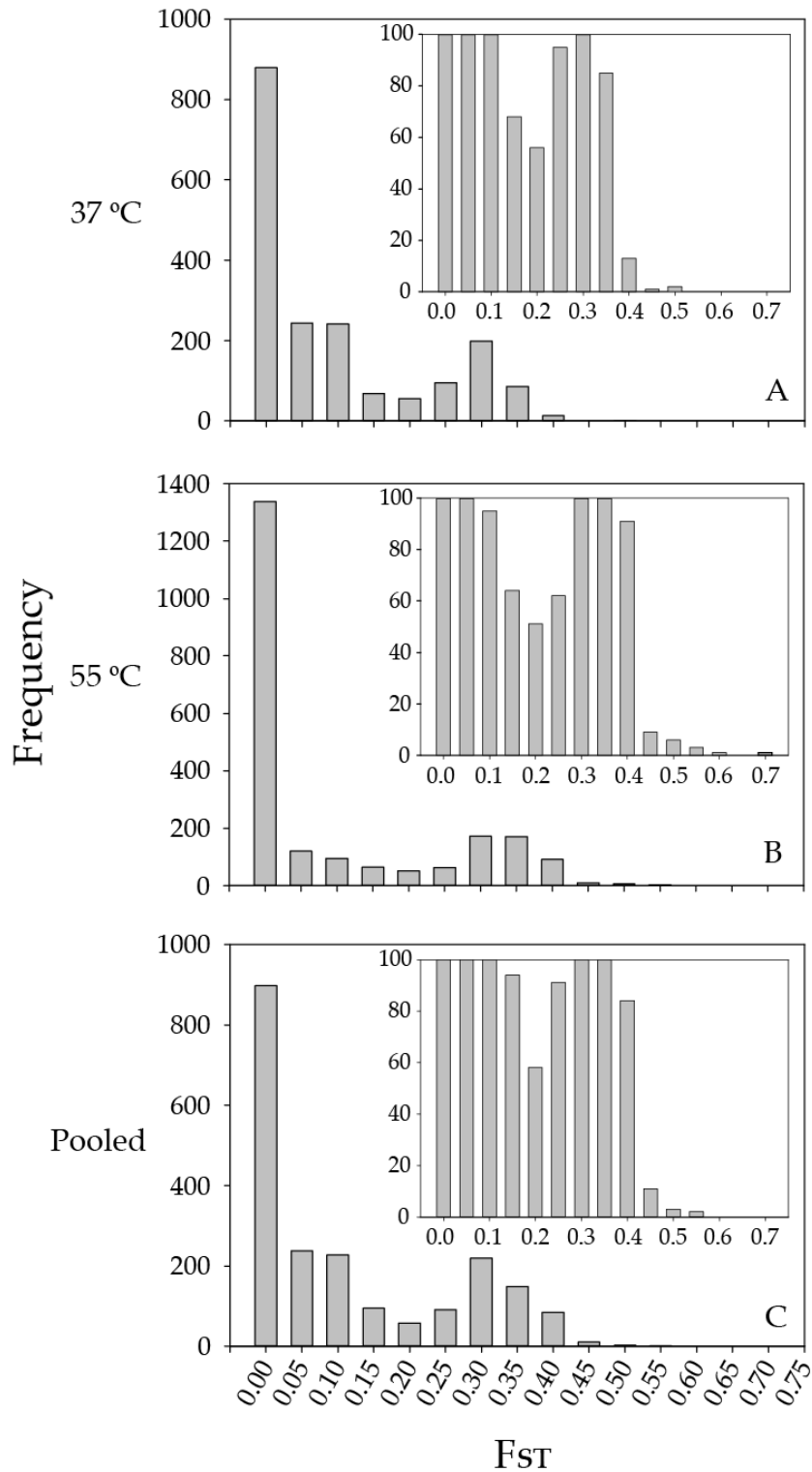
Gene	$D_{xy}$	Annotation
<b>167-28586</b>	<b>0.0161</b>	<b>Histidine kinase</b>
<b>77-48944</b>	<b>0.0088</b>	<b>ABC transporter</b>
33-9029	0.0057	Hypothetical Protein
<b>10-32834</b>	<b>0.0056</b>	<b>Urease alpha subunit</b>
93-17867	0.0054	Hypothetical Protein
<b>62-37089</b>	<b>0.0052</b>	<b>Chlorophyll A-B binding protein</b>
169-28586	0.0039	Hypothetical Protein
31-20539	0.0039	Glycosyl transferase family 2
<b>19-42545</b>	<b>0.0035</b>	<b>Hypothetical Protein</b>
84-29888	0.0034	Oxidoreductase domain protein
12-17867	0.0033	ABC-type $\text{NO}_3^-/\text{SO}_2\text{O}^-/\text{CHO}_3^-$ transport system
11-43020	0.0032	Function Unknown
12-14867	0.0031	Glycosyl transferase, group 1
111-46452	0.0031	HGT; MbtH domain protein
299-48944	0.0030	Putative peptidoglycan binding protein
<b>59-17867</b>	<b>0.0030</b>	<b>Hypothetical Protein</b>
92-17867	0.0029	Exodeoxyribonuclease 7 small subunit
80-29888	0.0029	Teichoic-acid-transporting ATPase
40-3504	0.0027	Hypothetical Protein
118-40954	0.0026	NAD(P)H-quinone oxidoreductase subunit 4



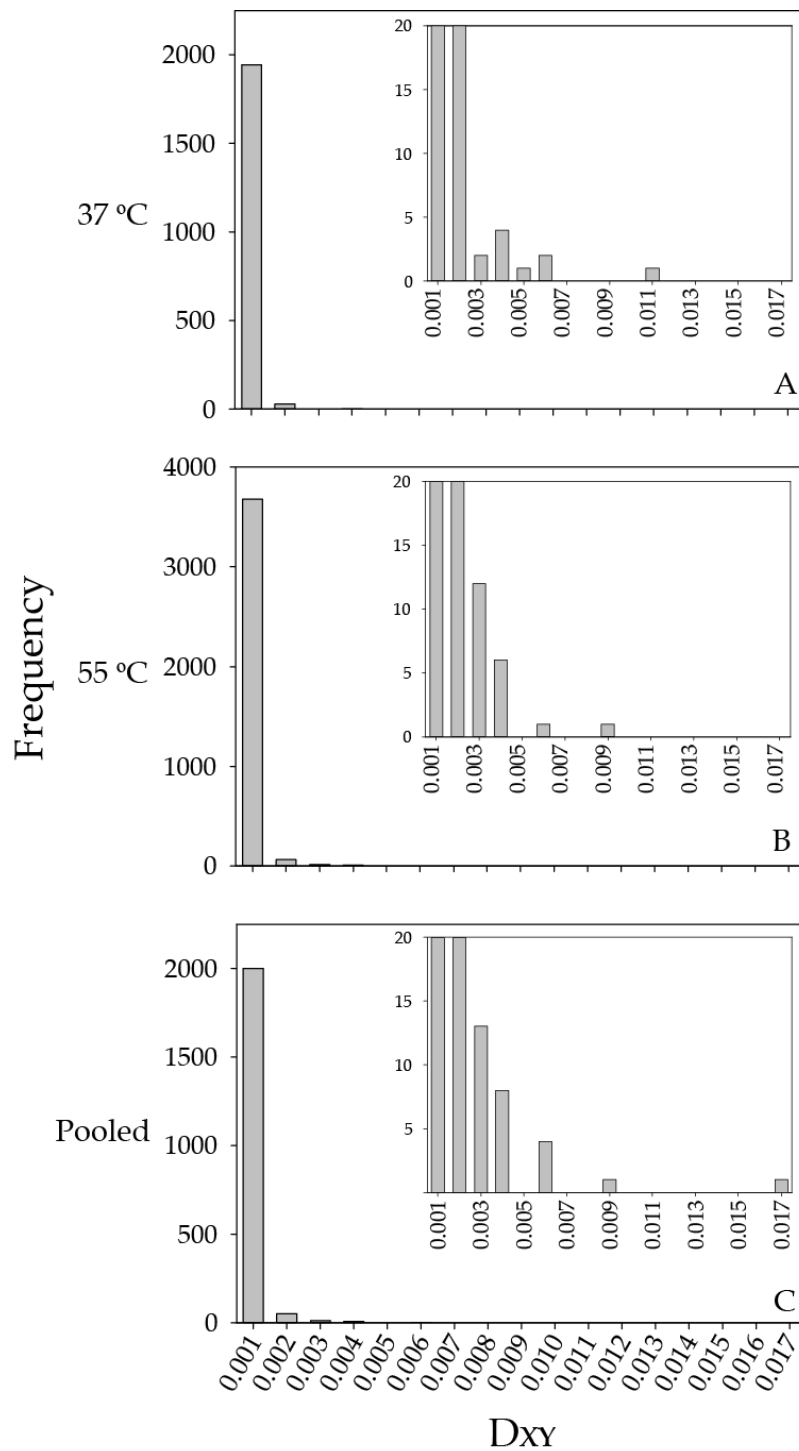


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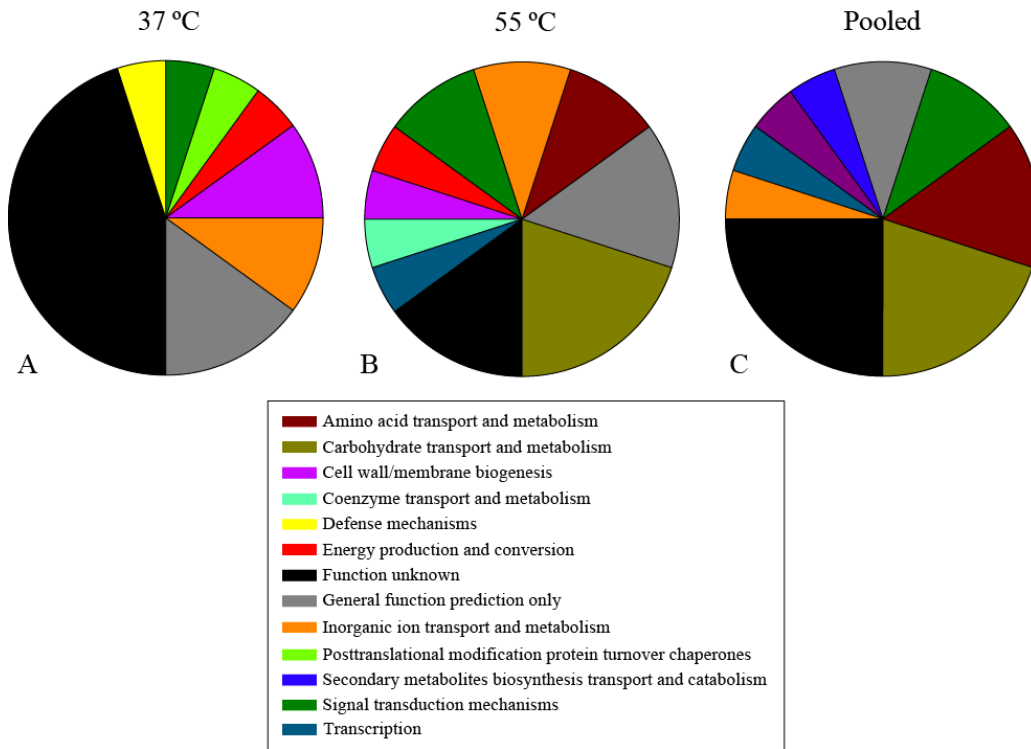
495 **Fig. 1.1** *M. laminosus* reaction norms for normalized ethylene production across temperature  
 496 treatments. Emboldened lines indicate a slope that is significantly different from zero at the 95%  
 497 confidence interval (ANOVA,  $p > 0.05$ ). Grey lines represent assayed strains for which slopes  
 498 were not significantly different from zero (NS). For clarity, error bars are not shown (see Table  
 499 1.1 for this information).  
 500



501  
 502 **Fig. 1.2** Relative genetic differentiation ( $F_{ST}$ ) between upper and lower phenotypic classes in the  
 503 37 °C (A), 55 °C (B), and pooled datasets (C). Insets have re-scaled views of the data in panels  
 504 A, B, and C to better visualize outlier values.  
 505

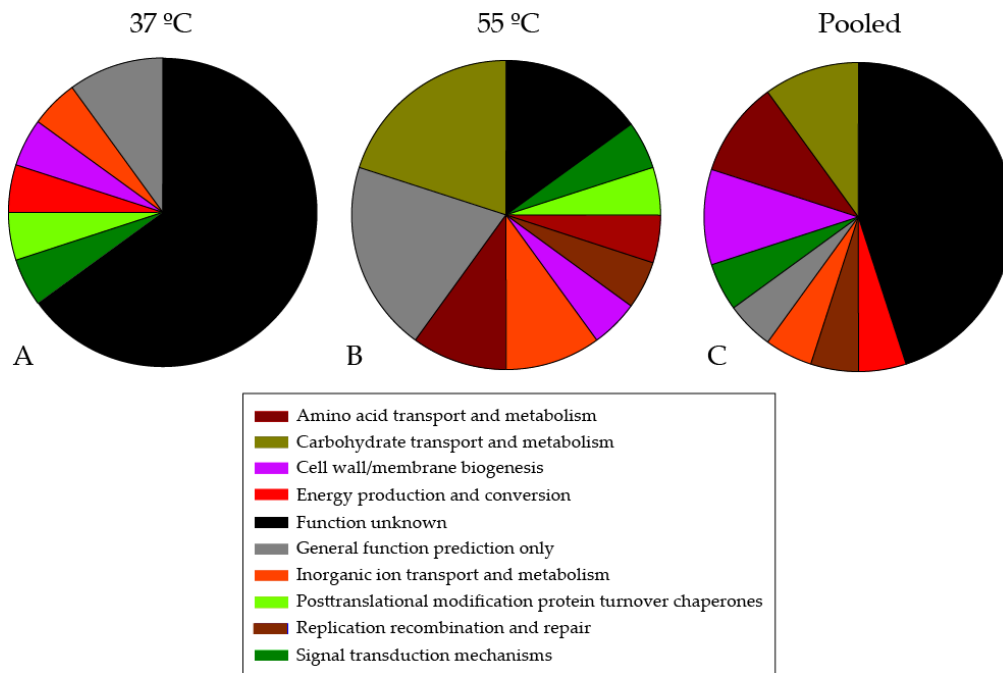


507 **Fig. 1.3** Absolute genetic differentiation ( $D_{XY}$ ) between upper and lower phenotypic classes in the  
 508 37 °C (A), 55 °C (B), and pooled datasets (C). Insets have re-scaled views of the data in panels  
 509 A, B, and C to better visualize outlier values.  
 510  
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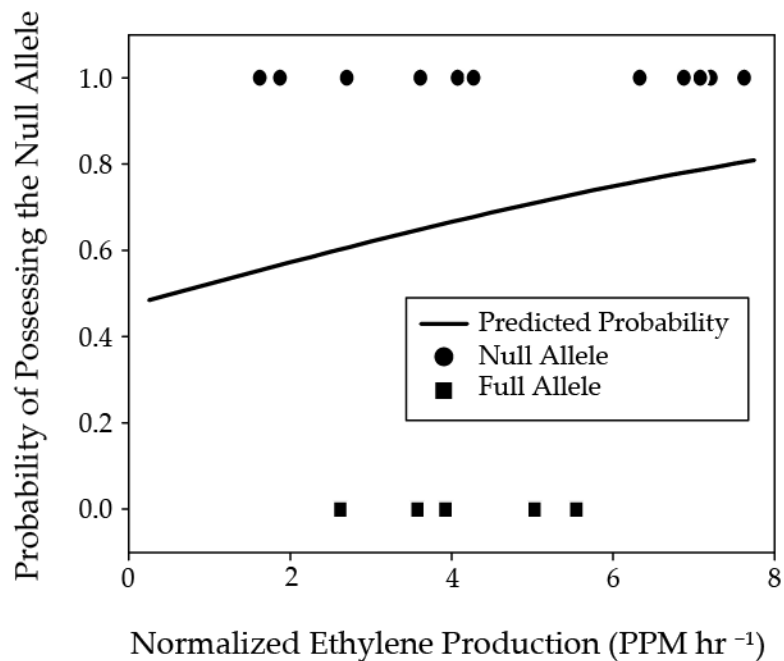
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**Fig. 1.4** Annotated clusters of orthologous groups (COG) categories for the top 1% of  $F_{ST}$  outlier loci in the 37 °C (A), 55 °C (B), and pooled datasets (C).



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**Fig. 1.5** Annotated clusters of orthologous groups (COG) categories for the top 1% of  $D_{XY}$  outlier loci in the 37 °C (A), 55 °C (B), and pooled datasets (C).



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**Fig 1.6** General linear model predicting the probability that a White Creek *M. laminosus* strain contains the premature stop codon at the 167-28586 locus based on strain mean normalized ethylene production. Circles are strain means for pooled ethylene production for strains with the premature stop codon and squares are strain means for those which have the full allele.

## Chapter 2

530

### 531 **Abstract**

532 In chapter 1, I reported that allelic variation at a histidine kinase gene (HK167-28586) was  
533 significantly associated with variation in nitrogen fixation performance in *M. laminosus* from a  
534 population at White Creek in Yellowstone NP. HK167-28586 also exhibits several molecular  
535 evolutionary signatures that suggest that allelic diversity at this locus encodes functionally  
536 important variation that has been maintained by some form of balancing selection. For there to  
537 be a phenotypic difference between allelic classes, I expect that the expression of the full and  
538 functional allele is required during either heterocyst development and/or steady state growth  
539 under nitrogen-limitation. Five different strains representing the three different alleles at the  
540 HK167-28586 locus that were observed in the White Creek population were tested in a simple  
541 expression assay under nitrogen-limitation using a reverse transcription polymerase chain  
542 reaction (RT-PCR) approach. Expression of the HK transcript was present at T<sub>0</sub> in all but one  
543 strain, and the transcript was not present in any samples at 48 hours after nitrogen step-down. I  
544 conclude that gene expression was turned off following heterocyst maturation and the onset of  
545 steady-state growth under nitrogen-limitation. More studies will be needed to assign a specific  
546 functional role to HK167-28586 and to determine the contribution of allelic variation at this locus  
547 to variation in nitrogen fixation.

548

### 549 **Introduction**

550 Two-component signal transduction systems (two component systems; TCSs) are the  
551 principal means by which bacteria sense and respond to environmental changes (Gao, Stock,  
552 2009; Wuichet *et al.*, 2010). TCSs are involved in a profound suite of critical cellular functions,  
553 including, but not limited to, chemotaxis, virulence, symbiosis, and carbon and nitrogen  
554 metabolisms (Parkinson, Kofoed, 1992). These signaling pathways can account for a significant  
555 proportion of bacterial genomes (up to ~2.5% in the cyanobacterium *Synechocystis sp.*; Mizuno *et*  
556 *al.* 1996) and have likely been crucial for bacterial adaptation.

557 Prototypical TCSs involve two separate proteins. A histidine kinase (HK), which usually  
558 has a sensory domain that interacts with the intra- or extracellular environment, serves as the  
559 input component of the system. The HK then transfers phosphoryl groups to a cognate response  
560 regulator (RR) to effect a change in gene expression or, sometimes, protein activity (Galperin,  
561 2010). Once stimulated, a well-conserved ATP binding domain at the C-terminal end of the HK  
562 catalyzes the autophosphorylation of a conserved histidine residue. The phosphorylated HK then  
563 transfers the His-bound phosphoryl group to an asparagine residue in a well-conserved receiver

564 domain on the RR. The phosphorylation of the receiver domain changes the structural  
565 conformation of a variable effector domain, which carries out the regulatory activity of the  
566 pathway. TCSs are often involved with several branching networks, yet operate with astounding  
567 fidelity (Laub, Goulian, 2007).

568 In chapter 1, I reported that allelic variation at a HK gene (HK167-28586) is associated  
569 with the ability of *M. laminosus* to fix nitrogen. Three alleles ranging in amino acid identity from  
570 96-99% were observed in the White Creek sample. These include: an allele with a nonsense  
571 mutation that is expected to eliminate 9 of the 17 predicted ATP binding residues in the encoded  
572 protein and therefore is expected to lack autophosphorylation and kinase activities; an allele that  
573 differs at 39 nucleotide positions; and an apparently rare recombinant null allele that is identical  
574 to the former at the 3' end and to the latter at the 5' end, and therefore contains the nonsense  
575 mutation. Because ATP hydrolysis is central to autophosphorylation and subsequent kinase  
576 activities, the loss of more than half of the ATP-binding pocket is expected to render the HK  
577 nonfunctional for these activities, even if it is expressed. For there to be a phenotypic difference  
578 between allelic classes, I further expect that expression of the full and functional allele is required  
579 either during heterocyst development and/or steady-state growth under nitrogen limitation. Here,  
580 this expectation is tested in a simple expression assay under nitrogen-limitation using a reverse  
581 transcription polymerase chain reaction (RT-PCR) approach.

582

## 583 **Methods**

### 584 *Culture Conditions and Sample Collection*

585 The assay was designed such that expression of HK167-28586 could be studied during  
586 both heterocyst development and subsequent steady-state growth under nitrogen-limitation. Five  
587 different strains representing the three different alleles at the HK167-28586 locus that were  
588 observed in the White Creek population were used in the experiment to determine whether each  
589 allele is transcribed. Strains WC119 and WC344 both have the full copy, while WC527 and

590 WC538 contain the null allele, and WC249 is the sole representative of the recombinant null  
591 allele. *M. laminosus* cells were grown in semi-continuous batch cultures in D medium  
592 (Castenholz, 1988) until ~5 mL of cell mass had accumulated for each strain. Just before the  
593 expression assay, the cells were washed twice in ND medium (D medium without combined  
594 nitrogen) by vortexing, centrifuging, and pouring off the supernatant before adding cells to  
595 triplicate flasks containing 250 mL of ND medium. Cultures were maintained at 37 °C with a  
596 12/12 hour light/dark cycle. The first cell sample was taken ~30 minutes after transfer to ND  
597 media (in the last hour of the dark cycle) and serves as the first time-point ( $T_0$ ).

598         Approximately 0.5 mL of cell mass was collected at 0, 6, 12, 18, 24, 36, and 48 hours  
599 after  $T_0$  using sterile Pasteur pipets and 2 mL microcentrifuge tubes. Samples were immediately  
600 immersed in liquid nitrogen and stored at -80 °C until extraction. A Qiagen RNeasy mini  
601 extraction kit was used to isolate RNA according to the manufacturer's instructions. Prior to  
602 constructing cDNA from RNA transcripts, the presence and quality of RNA was checked on a  
603 NanoDrop spectrometer, and DNA contamination of the RNA prep was screened via PCR using  
604 the primers and cycling conditions described below. A Thermo Scientific Maxima First Strand  
605 cDNA Synthesis Kit for RT-PCR was used to construct first strand cDNA according to the  
606 manufacturer's instructions. First strand synthesis was accompanied by a template-negative  
607 control.

#### 608 RT-PCR

609         HK cDNA was amplified by touchdown PCR on an MJR PTC-100 thermal cycler. The  
610 forward (5'-GGAATCCACCAACTATGG-3') and reverse (5'-CCAGGTGTAGAGTAGCAC-  
611 3') primers were designed manually. The resulting amplicon was 1025 bp in length and included  
612 the premature stop codon mutation of the putative null alleles. An initial denaturation step at 94  
613 °C for 3 min was followed by 30 cycles of 1 min at 94 °C, 30 sec at variable annealing  
614 temperatures, and 1 min at 72 °C. The initial annealing temperature was 54 °C and decreased  
615 every 10 cycles, reaching a final annealing temperature of 50 °C. A final extension phase at 72



616 °C for 3 minutes completed the program. PCRs were run with a template-negative and a positive  
617 control. Presence of the HK transcript at each time point was determined for each strain via gel  
618 electrophoresis of the cDNA amplicon. TAE gels consisted of 2% agarose and were run for ~15  
619 min at 98 V. Amplified DNA was stained using ethidium bromide and visualized on a UV  
620 transilluminator.

## 621 **Results**

### 622 **Results**

623 Expression of the HK transcript was present at T<sub>0</sub> in all but the strain WC344 samples,  
624 which first showed expression after 6 hours (Fig. 2.1). The first strain for which we could not  
625 detect the HK transcript was WC527 at 24 hours, though this may be due, in part, to the  
626 extremely low biomass left to harvest in this strain at that time point. The HK transcript was not  
627 present in any samples at 48 hours, and so appears to have been turned off somewhere between  
628 36-48 hours after nitrogen step-down. Though no quantitative estimates of heterocyst frequencies  
629 were made during this experiment, visual inspections of each strain at each time point suggest  
630 that all of the experimental strains reached their maximal heterocyst frequencies between 24-36  
631 hours after nitrogen depletion. This timeframe for heterocyst maturation is also corroborated by  
632 numerous other studies of heterocyst development in closely related cyanobacteria (Kumar *et al.*,  
633 2010; Wong, Meeks, 2001). In a subsequent expression assay under +N conditions (nitrate as N  
634 source), all three alleles of the gene were turned on in representative strains WC119, WC249, and  
635 WC344 (data not shown).

636

## 637 **Discussion**

638 Our results demonstrate that all three of the HK167-28586 alleles from the gene  
639 identified in Chapter 1 are expressed during *M. laminosus* heterocyst development and during +N  
640 growth. Although all three alleles are expressed, the alleles containing the premature stop codon  
641 are expected to be constitutively “off” because, without half of the ATP-binding sites,

642 autophosphorylation and kinase activities should be effectively nullified. Results from Chapter 1  
643 suggest that silencing HK167-28586 prior to steady-state growth under nitrogen limitation  
644 contributes to enhanced nitrogen fixation. This presents us with several important questions:  
645 what is the regulatory function of HK167-28586 (i.e., its cognate response regulator(s) and the  
646 transcriptional network in which it participates?); is it really a null allele, and what are the  
647 regulatory consequences of the elimination of much of the ATP-binding pocket?; and does the  
648 nonsense mutation come with a cost under certain environmental conditions?

649         The regulatory role of HK167-28586 cannot be discerned from this study. However, the  
650 HK167-28586 exhibits ~50% amino acid identity with three histidine kinases in the *Anabaena*  
651 PCC 7120 genome (alr1551, alr2739, and alr4882). For several reasons, alr4882 appears to be the  
652 ortholog. The HK gene is the same length as alr4882, which is not the case with other *Anabaena*  
653 homologs. Also, local gene order in the region is conserved: both HK167-28586 and alr4882 are  
654 downstream of a putatively orthologous annotated gene encoding a protein with a FIST domain  
655 (alr4881 and the corresponding *M. laminosus* gene are ~56% identical at the amino acid level).  
656 While FIST domains are phylogenetically widespread, they are biochemically uncharacterized,  
657 though they are predicted to bind small molecules (Borziak, Zhulin, 2007). In Mella-Herrera *et*  
658 *al.* (2011), alr4882 is referred to in unpublished data as a gene that is upregulated during  
659 heterocyst development 5-9 hours after nitrogen step-down. Insertional inactivation had no  
660 observed negative impact on -N growth, but their observations appear to be qualitative (the  
661 standard assay is to identify Fox- mutants by the yellowing of colonies on a plate) and don't  
662 speak to the fine-scale fitness effects that may be operating. Gene knock-outs in *Anabaena* PCC  
663 7120 and subsequent functional assays for nitrogen fixation may reveal differences in fitness that  
664 are too subtle for qualitative assays.

665         We expect that the premature stop codon nullifies the ability of HK167-28586 to function  
666 as a kinase. However, there are at least three possible scenarios where HK167-28586 could

667 continue to function in a TCS. First, the premature stop codon could be “leaky” and allow a full-  
668 length HK to be translated often enough to effect a regulatory response. Alternatively, though  
669 unlikely, the remaining ATP binding sites that are found upstream of the nonsense mutation may  
670 be sufficient to promote ATP-binding and autophosphorylation activity. To determine whether  
671 alleles with the premature stop codons have lost the ability to autophosphorylate, heterologously  
672 expressed protein can be assayed for autophosphorylation activity (Hastie *et al.*, 2006). Using  
673 this approach, the enzyme activity of each allelic variant of HK167-28586 could be compared  
674 quantitatively and with high sensitivity. In yet another scenario, the allele may have lost  
675 autophosphorylation activity but can still participate in the signal transduction network via  
676 phosphatase activity. Many histidine kinases are bifunctional enzymes that can phosphorylate as  
677 well as dephosphorylate their cognate response regulators (Alex, Simon, 1994). All of the White  
678 Creek *M. laminosus* alleles have an intact phosphotransfer domain, and, in at least one case, this  
679 domain alone is sufficient to support phosphatase activity of a histidine kinase (EnvZ; Zhu *et al.*,  
680 2000). Manipulation of the balance of kinase versus phosphatase activities might be an additional  
681 possible mechanism by which allelic variation at 167-28586 effects transcriptional rewiring via  
682 loss of function (Hottes, 2013).

683         Understanding the regulatory consequences of each HK167-28586 allele will be crucial  
684 in determining how genetic variation affects nitrogen fixation performance. Finding HK167-  
685 28586’s cognate response regulator(s) would make great strides in this regard. Phosphotransfer  
686 profiling (Skerker *et al.*, 2005) uses ATP radiolabelled with <sup>32</sup>P and SDS-PAGE to identify  
687 phosphotransfer events between purified HKs and RRs. This approach is necessary for  
688 identifying “orphans”, or HK-RR pairs that are not expressed under the same operon, as is the  
689 case here. The differences in the transcriptome among strains with different alleles identified in  
690 RNA-seq data would likely yield insight into the downstream regulatory consequences of each  
691 HK167-28586 allele and help guide more directed functional assays under varying environmental

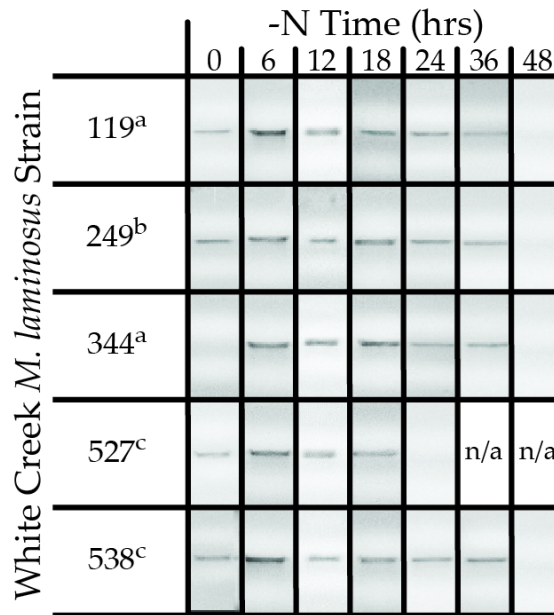
692 conditions. Transcriptomic comparisons between allele variants would help to identify any  
693 regulatory networks that have been altered as a result of the nonsense mutation. Furthermore, the  
694 existence of a recombinant null allele may enable us to parse the individual regulatory effects of  
695 the nonsense mutation and the highly polymorphic region at the 5' end of the gene. It is possible  
696 that the null allele results in a fitness trade-off under varying environmental conditions. A  
697 transcriptomic approach could also be used to compare differences in gene regulation for each  
698 allele under varying environmental conditions. Investigations such as these will inform our  
699 understanding of the role that balancing selection may play in the maintenance of the HK167-  
700 28586 alleles.

## 701 **Conclusion**

702 All three HK167-28586 alleles were expressed during nitrogen-replete conditions and  
703 during heterocyst development under nitrogen-limitation. Thus, I expect there to be differences in  
704 the transcriptional profiles of strains with functional and putative null alleles, respectively. This  
705 is the first step in demonstrating a functional basis for the pattern of association with improved  
706 nitrogen fixation in *M. laminosus* from Chapter 1. However, more studies will be needed to be  
707 able to assign a functional role to HK167-28586 and to determine the underlying cause of  
708 variation in nitrogen fixation for each allele.

709

710 **Figures**



711

712 **Fig. 2.1** Presence or absence of a HK167-28586 transcript after nitrogen step-down in five *M.*  
 713 *lamosus* strains from White Creek. Subscripts next to strain numbers indicate which allele the  
 714 strain contains (“a” is the null allele, “b” is the recombinant null allele, and “c” is full the copy  
 715 allele).

716

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