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Mutualism variation in the nodulation response to nitrate

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Abstract

The evolution of mutualisms under novel selective pressures will play a key role in ecosystem responses to environmental change. Because fixed nitrogen is traded in plant–rhizobium mutualisms, increasing *N* availability in the soil is predicted to alter coevolution of these interactions. Legumes typically decrease the number of associations (nodules) with rhizobia in response to nitrate, but the evolutionary dynamics of this response remain unknown. We grew plant and rhizobium genotype combinations in three *N* environments to assess the coevolutionary potential of the nodule nitrate response in natural communities of plants and rhizobia. We found evidence for coevolutionary genetic variation for nodulation in response to nitrate (G × G × E interaction), suggesting that the mutualism response to *N* deposition will depend on the combination of partner genotypes. Thus, the nitrate response is not a fixed mechanism in plant–rhizobium symbioses, but instead is potentially subject to natural selection and dynamic coevolution.

Introduction

Coevolution is dynamic and depends on the abiotic and biotic environment (Stinchcombe & Rausher, 2001, 2002; Thompson, 2005, 2009). Consequently, the selective landscape for coevolving interactions is likely being altered by global environmental change, which includes multiple abiotic and biotic forces (e.g. rising temperatures, invasive species, nitrogen deposition). Recently documented effects include phenological mismatch in plant–pollinator interactions (Gordo & Sanz, 2005) and the disassociation of coral symbionts (Glynn & D'Croz, 1990). Symbiotic mutualisms are keystones in structuring natural communities (Janzen, 1985), and because of their inherent instability may be particularly susceptible to environmental change (as recently argued by Six, 2009), yet we know little about how they will respond to these pressures.

One mutualism of global ecological and economic impact occurs between legumes (Fabaceae) and nitrogen (N)-fixing rhizobia. Rhizobia live in symbiotic nodules on the roots of host plants and therein fix atmospheric dinitrogen to plant-available ammonium in return for plant photosynthates. As a result of their mutualism, plants and rhizobia are responsible for the majority of (non-anthropogenic) fixed *N* in terrestrial ecosystems and account for 27% of global crop production (Graham & Vance, 2003). Changing the abundance of a traded resource in mutualism can shift ecological interactions from mutualism to parasitism (Neuhauser & Fargione, 2004) or impose selection for less-beneficial mutualists (West *et al.*, 2002); therefore, anthropogenic increases in the terrestrial supply of plant-available *N* (Vitousek *et al.*, 1997; Vergeer *et al.*, 2008) are expected to alter legume–rhizobium coevolution.

One trait potentially important in legume-rhizobium coevolution is the number of root nodules formed. Nodule number is truly a trait of the symbiosis, as the phenotype is determined by the plant genotype × rhizobium genotype interaction (G × G; Heath & Tiffin, 2007; Heath, 2010). Nodule number is likely the main determinant of rhizobium fitness during the symbiotic stage of the life cycle (Heath & Tiffin, 2009), and nodulation by rhizobia is necessary for the establishment of many legume species in natural communities (Parker

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et al., 2006). Therefore, the degree of nodulation is a likely target of selection. Nodulation is not, however, equally beneficial for plants in all environments. Nodulation is costly for plants (Layzell *et al.*, 1981); therefore, legumes should avoid symbiosis when *N* is readily available. In fact, decreases in nodulation in the presence of nitrate (hereafter simply the nitrate response) have long been appreciated (Streeter, 1988). Much work has focused on the physiology underlying nodulation and the nitrate response (recently reviewed by Glyan'ko *et al.*, 2009) and on the agronomic goal of reducing this response through breeding (e.g. Pate & Dart, 1961; Evans, 1982). Despite the common assertion that the nitrate response might be an adaptation to N-rich environments (e.g. Caetano-Anollés & Gresshoff, 1991), however, this trait has received little evolutionary attention.

Nodulation is also a trait likely to experience a selection mosaic (*sensu* Thompson, 2005). Selection mosaics in species interactions can occur when selection on the traits mediating coevolution varies with the abiotic or biotic environment, resulting in variation in coevolutionary interactions in time or space (Thompson, 2005). Selection mosaics can be defined as a genotype × genotype × environment (G × G × E) interaction for fitness (Piculell *et al.*, 2008; Thompson, 2009; also see Gomulkiewicz *et al.*, 2007 for discussion). Geographical variation in nitrogen deposition has been suggested to result in plant local adaptation to *N* availability (e.g. Vergeer *et al.*, 2008). Likewise, variation in *N* abundance might be expected to drive spatially variable outcomes of coevolution in the legume–rhizobium symbiosis, if *N* availability shifts selection on one or both partners in the interaction. Tests for G × G × *N* interactions thus reveal the potential for nitrogen-mediated selection mosaics in plant–rhizobial mutualisms.

The nitrate response of nodulation is likely particularly important for understanding how increased global *N* will influence the persistence of legume–rhizobium mutualisms. Using the model symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*, we studied the response of plant and rhizobium genotypes collected from natural populations in their native range to each of three different *N* environments to determine (i) whether there is coevolutionary genetic variation in natural communities of plants and rhizobia for the nitrate response of nodulation and (ii) how this variation influences plant growth in different *N* environments.

Materials and methods

Experiment

We grew three plant genotypes in each of four rhizobium environments (three strains, plus an uninoculated control treatment), in each of three nitrate levels in a factorial design in the greenhouse (with five replicates, for a total of 180 plants), and estimated the effects of symbiosis on plant and rhizobium growth and fitness benefits. We focused on nitrate levels known to alter, but not eliminate, nodulation in nitrate-sensitive *M. truncatula* (Ewing & Robson, 1990; Fei & Vessey, 2009). Nitrate levels in nature are highly spatially and temporally variable and not reliably predicted from deposition rates (Kristensen *et al.*, 2004); however, our high nitrate level (1 mM NO_3, or 14 ppm NO_3–N) is within the range of soil solution *N* levels near fertilized agricultural fields (Brown *et al.*, 1993; Kanwar *et al.*, 2006), thus simulating an environment under heavy *N* deposition. We chose three plant maternal families (*Chat 1*, *Chat 3* and *Sals 1*) and three rhizobium strains (*Chat c*, *Sals b* and *Naut a*) from natural populations (*Chat*, *Naut* and *Sals*) in the native range on the Mediterranean coast of France (detailed in Heath, 2010). These genotypes were previously shown to exhibit G × G interactions for plant and rhizobium fitness benefits; however, previous analyses found no evidence for coadaptation of local plant and rhizobium combinations (Heath, 2010).

Preparation of seedlings and rhizobium inoculum was as previously described (Heath, 2010). Seedlings of the appropriate plant genotype were transplanted into modified leonard jars (Vincent, 1970). These pots were fashioned by nesting two Magenta tissue culture boxes (Magenta Corp, Rockville MD, USA): the upper box containing soil mix and roots was nested inside a lower box containing nutrient solution, with a 100% wool felt wick immersed in the solution below. Nutrient solution was quarter-strength Fåhraeus (Somasegaran & Hoben, 1994) adjusted to the correct *N* concentration (low = 0 mM, intermediate = 0.1 mM and high = 1.0 mM added KNO_3). Magenta pots, each containing a 4:1 mix of Turface MVP (Profile Products LLC, Buffalo Grove, IL, USA) and Sunshine Mix #2 (Sun Gro, Bellevue, WA, USA), were soaked in nutrient solution prior to transplant, at which time seedlings were also inoculated by pipetting 1 mL (10^5 cells) diluted rhizobium culture at their base. Plants were grown in 16-h light/8-h dark photoperiod in the greenhouse and watered by refilling the bottom box with nutrient solution as necessary. The 45 uninoculated plants remained uncontaminated, with a single exception, which had five nodules.

Data and analysis

While our primary trait of interest is nodule number, we also collected and analysed data on other plant traits likely to respond to *N* and changes in nodulation. We determined plant height and leaf number for the 169 surviving plants 2 weeks post-transplant. At harvest (9 weeks post-transplant), we measured aboveground and belowground biomass, fruit number and nodule number for the surviving 161 plants. Nodule number was scored as zero if no nodules were present, and these data were included in all analyses. We estimated nodule size

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as the average length of all nodules on each of the 60 nodulated plants, as not all experimental plants formed nodules; nodule length for non-nodulated plants was treated as missing data. Dependent variables were analysed using PROC GLM in SAS (SAS Institute, Cary, NC, USA), with plant genotype (family), rhizobium genotype (strain) and *N* level (nitrogen) and all possible interactions included as fixed effects. Dependent variables were resistant to normalization by transformation; for all hypothesis tests, we present parametric *F* statistics but with significance tests determined from a randomization test with 10 000 replicates, implemented as previously described (Cassell, 2002; Heath, 2010).

Recently, the concept of the false discovery rate (FDR) has been recommended as an appropriate method for interpreting table-wide or experiment-wide rates of Type I error while preserving statistical power (Storey & Tibshirani, 2003; Verhoeven *et al.*, 2005). Because we used the same general statistical model for more than one dependent variable, our hypothesis tests of model effects can be aided by consideration of both their *P*-value as well as their *Q*-value. For a given test, the *P*-value represents the chance of that effect being deemed significant when truly null, whereas the *Q*-value indicates the per cent of *statistically significant* tests that are expected to be false discoveries when interpreting that test as statistically significant (Storey & Tibshirani, 2003). We estimated *Q*-values using the QVALUE software (Storey, 2002).

Results

Nitrate responses

Plant families, rhizobium strains and the G × G interaction each determined, in part, the number of nodules formed in symbiosis (family, strain and family × strain interaction, Table 1). Surprisingly, we found no significant main effect of nitrate addition on nodulation. Instead, the nitrate response was mediated by the rhizobium genotype (strain × nitrogen interaction, Table 1, Fig. 1). These interactions suggest that the nitrate environment leading to greatest nodulation depends on rhizobium genotype (and vice-versa). On average, strain *Naut a* formed fewer nodules in the nitrate-addition treatments, whereas inoculation by strain *Chat c* actually increased nodulation (Fig. 1).

The nitrate effect was also mediated by the compatibility between plant and rhizobium genotypes (i.e. G × G × E, or family × strain × nitrogen interaction, Table 1). Although the significance of the G × G × E term is on the border of significance, the *Q*-value suggests interpreting it as significant would incur few false discoveries–5.1% of the significant results in Table 1 (i.e. fewer than 1 of the 12 significant tests). The three-way interaction indicates that the plant genotype–rhizobial genotype combinations that led to greatest nodulation differed

Table 1 ANOVA results for plant growth and nodule traits of three *M. truncatula* families with each of three *S. meliloti* strains in each of three nitrate treatments (0, 0.1 and 1.0 mM KNO_3). *P*-values presented are from randomization test.

	Plant Biomass			Leaf number			Fruits			Nodule number			Nodule size		
Source	*F*	*P*	*Q*	*F*	*P*	*Q*	*F*	*P*	*Q*	*F*	*P*	*Q*	*F*	*P*	*Q*
Family	$F_{2,\ 125} = 14.37$	0.0001	0.0002	$F_{2,\ 132} = 13.79$	0.0001	0.0002	$F_{2,\ 125} = 60.39$	0.0001	0.0002	$F_{2,\ 125} = 10.24$	0.0001	0.0002	$F_{2,\ 35} = 1.43$	0.2591	0.1477
Strain	$F_{3,\ 125} = 2.05$	0.1138	0.0865	$F_{3,\ 132} = 3.79$	0.0115	0.0202	$F_{3,\ 125} = 0.69$	0.5667	0.2294	$F_{3,\ 125} = 10.71$	0.0001	0.0002	$F_{3,\ 35} = 1.98$	0.1386	0.0929
Nitrogen	$F_{2,\ 125} = 4.45$	0.0142	0.0202	$F_{2,\ 132} = 3.36$	0.0370	0.0422	$F_{2,\ 125} = 2.61$	0.0785	0.0639	$F_{2,\ 125} = 0.18$	0.8353	0.2885	$F_{2,\ 35} = 0.13$	0.8766	0.2939
Family × Strain	$F_{6,\ 125} = 0.98$	0.4394	0.2003	$F_{6,\ 132} = 1.02$	0.4224	0.2003	$F_{6,\ 125} = 0.66$	0.6867	0.2525	$F_{6,\ 125} = 2.85$	0.0128	0.0202	$F_{6,\ 35} = 0.84$	0.5104	0.2155
Family × Nitrogen	$F_{4,\ 125} = 0.69$	0.6037	0.2294	$F_{4,\ 132} = 1.09$	0.3715	0.1841	$F_{4,\ 125} = 1.68$	0.1557	0.0986	$F_{4,\ 125} = 2.21$	0.0750	0.0639	$F_{4,\ 35} = 0.70$	0.5917	0.2294
Strain × Nitrogen	$F_{6,\ 125} = 1.73$	0.1229	0.0876	$F_{6,\ 132} = 2.15$	0.0501	0.0519	$F_{6,\ 125} = 1.19$	0.3099	0.1682	$F_{6,\ 125} = 2.44$	0.0272	0.0344	$F_{6,\ 35} = 0.85$	0.5064	0.2155
Family × Strain × Nitrogen	$F_{12,\ 125} = 1.14$	0.3301	0.1710	$F_{12,\ 132} = 0.42$	0.9531	0.3104	$F_{12,\ 125} = 0.72$	0.7406	0.2638	$F_{12,\ 125} = 1.81$	0.0546	0.0519	$F_{12,\ 35} = 1.44$	0.2432	0.1459

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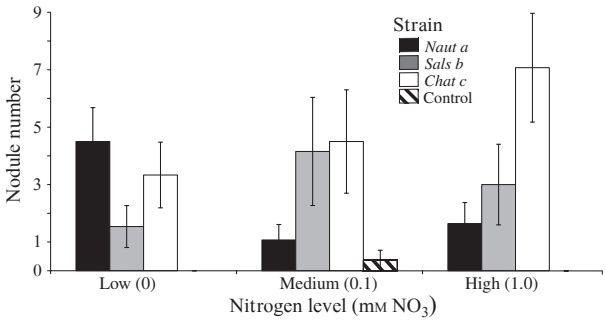

Fig. 1 Number of nodules formed by three *S. meliloti* strains in each of three nitrate environments. Raw means (± SE) presented.

depending on the nitrate environment (Fig. 2). While nodulation was restricted in high nitrate when plant genotype *Chat 3* interacted with rhizobium genotype *Chat c*, nodulation increased with high nitrate when plant genotype *Sals 1* interacted with rhizobium *Chat c* (Fig. 2). Therefore, while nodulation responded to nitrate addition in this experiment, the response was genetically variable and depended on the combination of partner genotypes in the symbiosis. In an analysis of specific nodulation, trends were very similar (nodules per root biomass; results not shown).

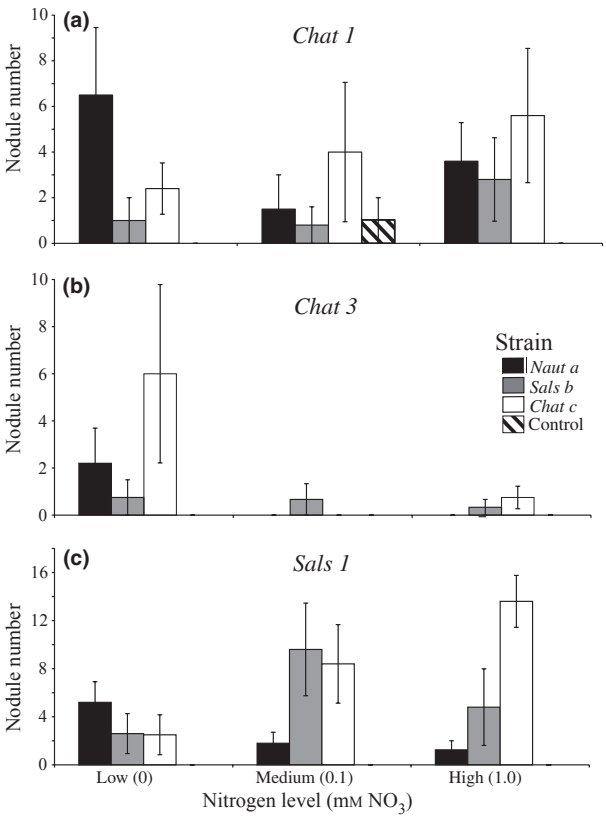

Fig. 2 Number of nodules formed by *M. truncatula* family *Chat 1* (a), *Chat 3* (b) and *Sals 1* (c) in symbiosis with each of three *S. meliloti* strains (or uninoculated controls), in each of three nitrate environments. Raw means (± SE) presented.

Plant growth

As expected, we found a significant effect of nitrate addition on early leaf number and total plant biomass (though nitrate did not significantly affect fruit number; $P = 0.08$, Table 1). Plants had on average 7.6 ± 0.3 ($\bar{x}$ ± 1 SE) leaves in high nitrate, compared to 6.5 ± 0.3 and 7.31 ± 0.3 leaves in medium and low nitrate, respectively. Similarly, plants weighed 0.84 ± 0.07 g in high nitrate, compared to 0.54 ± 0.07 and 0.62 ± 0.07 g in medium and low nitrate, respectively. Therefore, plants in the intermediate *N* treatment actually performed slightly worse than plants in the high and low *N* treatments. However, after Tukey's correction for multiple means, plants in the high *N* treatment had significantly more leaves ($P = 0.04$) and biomass ($P = 0.01$) than did plants in the intermediate treatment, whereas low and intermediate treatments were not significantly different from each other (all $P > 0.16$). Plant biomass and early leaf number were both positively correlated with nodule number ($r_{161} = 0.59$, $P < 0.0001$ and $r_{161} = 0.28$, $P = 0.0004$, respectively), although fruit number was not ($P = 0.14$). Nevertheless, the inclusion of nodule number as a covariate in analyses of plant biomass and leaf number did not alter the significance, nor the direction, of the effects of nitrate treatments (results not shown). Moreover, there was no main effect of nitrate treatment on nodule number in our original analyses ($P = 0.84$, Table 1). Collectively, these lines of evidence suggest that changes in nodule number do not explain the main effect of *N* treatments on plant fitness components, but instead implicate increased plant performance in high *N* because of the direct benefits of soil nitrate.

There was also suggestion that the early response of plant growth to nitrate depended on the strain with which they formed symbiosis (strain x nitrogen interaction for early leaf number, Table 1, Fig. S1), although this effect was not detected for total plant biomass or fruit number (Table 1, Fig. S2). Inclusion of nodule number as a covariate in the analysis of leaf number rendered the strain x nitrogen term non-significant ($P = 0.27$), suggesting that the strain x nitrogen interaction for leaf number is explained by variation in nodule number (because nodule number and leaf number were positively correlated, see above). We did not detect evidence of G × G × E interaction for any measure of plant performance (Table 1).

Discussion

How mutualisms coevolve in response to the changing environment will play an important role in determining

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the ultimate impacts – both ecological and economic – of anthropogenic changes such as increased *N* deposition. Two major results emerged from our experiment. First, we found evidence that rhizobium genotype by nitrogen and plant genotype by rhizobium genotype by nitrogen interactions (i.e. G × E and G × G × E interactions) mediate the symbiotic response of plants and rhizobia to different *N* environments. Thus, the nitrate response of plant-rhizobium nodulation is not a fixed mechanism, but is better viewed as a dynamic trait – i.e. an 'extended phenotype' (Dawkins, 1989) or emergent property of symbiosis – and therefore potentially subject to coevolutionary selection. Second, the presence of G × G × E interaction, which in essence defines a selection mosaic (Piculell *et al.*, 2008), suggests that geographic variation in *N* abundance could lead to spatially structured coevolution in this symbiosis. We discuss both of these results below in the context of the maintenance of mutualisms in response to *N* deposition and the geographic mosaic theory of coevolution, respectively.

Mutualism maintenance in the face of *N* deposition

What does coevolutionary genetic variation (i.e. G × G) for the nitrate response mean for mutualism stability in the face of *N* deposition? If increases in nitrate resulted universally in decreased or abolished nodulation, then we might expect that anthropogenic *N* deposition should eventually result in mutualism breakdown (Sachs & Simms, 2006). While this prediction is consistent with the results from some genotype combinations in our study, we also found the opposite pattern. For example, by analyzing each plant family separately (see Table 2), we found that the results for each family would lead to different predictions for mutualism persistence. Nodulation by plant family *Chat 1* was not affected significantly by *N* or its interaction with strain (all $P > 0.4$), and therefore the stability or persistence of mutualism would not be predicted to change with the nitrate environment. By contrast, nodulation by family *Chat 3* was very sensitive to nitrate (Table 2); few nodules were formed with any strain in increased nitrate environments – potentially suggesting that nitrate might contribute to disassociation of these mutualist genotypes and mutualism breakdown. Finally, while there was no change in the mean number of nodules formed by family *Sals 1* across nitrate environments, the number of nodules formed with different strains did change (strain × nitrogen interaction, Table 2). Such a result would suggest that alterations to the *N* environment might shift *Sals 1*-mediated selection on rhizobium populations. Our analysis is not intended to make specific quantitative predictions for any population or genotype *per se*. Rather, taken together, these patterns of G × E, G × G and G × G × E interactions for mutualism traits suggest that genetic variation for symbiotic responses to *N* is abundant and will be important in mediating the consequences of global change for this important mutualism.

While the G × G × E interaction appeared important for determining rhizobium nodule numbers and therefore the benefits to rhizobia, it did not significantly affect plant fitness components. Moreover, the strain × nitrogen interaction that we did detect for leaf number appeared to be mediated by changes in nodule number and their positive feedback on plant growth. Overall, our results suggest that nodulation was most sensitive to the interactive effects of genetic and environmental variation in our experiment, and therefore highlight the lability and context dependence of symbiotically determined traits such as nodule number. More generally, our results underline the difficulty of teasing apart fitness effects for host and symbiont in an intimate mutualism, in which the fitness of partners covaries.

Potential for geographic mosaics of symbiosis

An important component of the geographic mosaic of coevolution (Thompson, 2005) is the prediction that species interactions experience selection mosaics, which in turn lead to geographical variation in the outcomes of trait evolution. Selection analyses have been used to demonstrate geographically variable and environmentally dependent natural selection in other systems (e.g. Benkman *et al.*, 2003; Rudgers & Strauss, 2004); however, mosaics differ from variable selection (i.e. geographically or environmentally variable selection gradients), in that they require that the environment (biotic or abiotic) changes the fitness effects of the interaction between a given pair of genotypes (Gomulkiewicz *et al.*, 2007). Despite intensive work on coevolution (Thompson, 2005, 2009), we still have relatively few studies in which the genetic (as opposed

Table 2 ANOVA results of nodule number for each of the three *M. truncatula* families in this experiment. Each family was grown with each of three *S. meliloti* strains in each of three nitrate treatments (0, 0.1 and 1.0 mM KNO_3). *P*-values presented are from randomization test.

	Plant family					
	Chat 1		*Chat 3*		*Sals 1*	
Source	*F*	*P*	*F*	*P*	*F*	*P*
Strain	$F_{3, 43} = 3.44$	0.0260	$F_{3, 36} = 2.57$	0.0580	$F_{3, 46} = 8.53$	0.0020
Nitrogen	$F_{2, 43} = 0.48$	0.6520	$F_{2, 36} = 5.28$	0.0080	$F_{2, 46} = 1.66$	0.2190
Strain × Nitrogen	$F_{6, 43} = 1.02$	0.4620	$F_{6, 36} = 2.00$	0.0760	$F_{6, 46} = 3.02$	0.0130

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to environmental or ecological) components of an interaction have been teased apart (see Tétard-Jones *et al.*, 2007 and Vale & Little, 2009 for examples).

If plant G × rhizobium G × *N* interactions drive a geographic mosaic of coevolution in legume–rhizobium symbiosis, then two conditions are required: *N* must be spatially variable, and among-population gene flow must be somewhat restricted (Nuismer *et al.*, 1999; Gomulkiewicz *et al.*, 2007). European *N* deposition varies at a broad scale latitudinally, as well as at a local scale dependent on agricultural proximity and precipitation (Vergeer *et al.*, 2008). These observations, combined with many studies documenting naturally occurring variation in *N* abundance (e.g. Holdensen *et al.*, 2007), suggest that *N* variation is prevalent at even fine spatial scales. Populations of *M. truncatula* are well known to be genetically structured (e.g. as indicated by F_{ST}; Bonnin *et al.*, 1996), and past work has demonstrated population-level quantitative genetic differentiation in mutualism traits, such as nodule number (Heath, 2010). The extent of population structure and spatial scale of gene flow in *S. meliloti* have yet to be thoroughly resolved because studies of diversity in the native range have focused on variation either among strains from a single location (e.g. Bailly *et al.*, 2006) or among a haphazard collection of isolates from different locations (e.g. Eardly *et al.*, 1990; Biondi *et al.*, 2003). However, in southern France (the source of our plant and rhizobium genotypes), both neutral markers and symbiotic phenotypes indicated differentiation among a small number of soil populations (Heath, 2010). Collectively, these data suggest that symbiotic responses to abiotic *N* have the potential to shape geographical patterns in legume–rhizobium interactions.

Conclusions

While our results are limited to the particular genotypes, nitrate levels and environmental conditions in this experiment, they nevertheless suggest that the rate or even direction of coevolutionary change in response to increasing global *N* might depend on the particular combinations of mutualist genotypes. Similarly, our results suggest that geographic variation in *N* abundance could lead to appreciable spatial structure of symbiotic coevolution between plants and legumes. Experiments involving a larger sample of plants and rhizobia to long-term nitrate fertilization treatments under more realistic, field conditions will ultimately be necessary to understand how these mutualisms are adapting, and will continue to adapt, to global environmental change.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Mean leaf number of *M. truncatula* plants in symbiosis with each of three *S. meliloti* strains (or uninoculated controls), in each of three nitrate treatments.

Figure S2 Mean biomass (*g*) of *M. truncatula* plants in symbiosis with each of three *S. meliloti* strains (or uninoculated controls), in each of three nitrate treatments.

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