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Drivers of Symbiotic Quality in Wild *Bradyrhizobium*

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ABSTRACT OF THE DISSERTATION

Drivers of Symbiotic Quality in Wild *Bradyrhizobium*

by

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Understanding the drivers of variation in symbiont quality is a fundamental objective in the study of mutualisms. Eukaryotic hosts express control traits that can selectively favor beneficial symbionts over ineffective genotypes, but bacterial symbionts range widely in beneficial quality. Evolutionary instability in symbiotic function and/or context dependency in the expression of symbiotic traits are predicted to contribute to this variation.

The *Acmispon-Bradyrhizobium* mutualism is a model system for studying variation in symbiotic traits. *Acmispon* hosts sanction ineffective symbionts, yet *Bradyrhizobium* naturally vary in symbiotic function. However, the incidence, distribution, and evolution of symbiotic quality in *Bradyrhizobium* from natural habitats remains unclear. I investigated the evolution and spatial distribution of *Bradyrhizobium* symbiotic effectiveness across a metapopulation of *A. strigosus* hosts. Symbiotic quality was evolutionarily unstable, consistent with the repeated evolution of non-nitrogen fixing

Bradyrhizobium, and suggests that the loss of nitrogen fixation may be a common process.

To examine if symbiotic ineffectiveness was expressed as a maladapted, context dependent outcome, I quantified fitness and fitness-effects of diverse *Bradyrhizobium* isolates on sympatric and allopatric *Acmispon* hosts. Several isolates were found to be symbiotically ineffective and rhizobial fitness proxies uncovered evidence of rhizobial exploitation. This data suggests that host exploitation also maintains ineffective rhizobia, and thus overall variation in symbiont quality in natural populations.

Symbiotic quality can also vary due to biotic environment, and although rhizobia are best known for symbiotic function, the majority of rhizobia are non-symbiotic. To test if non-symbiotic conspecifics effect the *Acmispon-Bradyrhizobium* mutualism, I coinoculated hosts with mixtures of symbiotic and non-symbiotic *Bradyrhizobium*. In most cases, the presence of non-symbiotic *Bradyrhizobium* reduced host and symbiont performance and data suggests this occurs via competitive interactions at the root-soil interface.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GENERAL INTRODUCTION

A fundamental objective in the study of mutualisms is to understand what drives variation in symbiont quality. Bacterial mutualisms with eukaryotic hosts are intimate, reciprocally beneficial interactions (Douglas 2010). Eukaryotic hosts most often infectiously acquire symbionts from the environment (i.e., horizontal transmission) (Sachs *et al.* 2011) and after infection, bacterial partners provide critical benefits to hosts, including enhanced growth, tolerance to stress, and protection from predators and pathogens (Mueller & Sachs 2015). Despite host mechanisms that select for mutualism stability, such as the ability to selectively favor beneficial genotypes (Bronstein 1994b; Sachs *et al.* 2004), bacterial partners range from highly beneficial to ineffective (Bronstein 1994a).

Variation in symbiotic quality could be driven by evolutionary instability in symbiotic function and/or context dependency in the expression of symbiotic traits in space or time (Bronstein 1994a; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath & Stinchcombe 2014). Bacterial symbionts have a substantial evolutionary advantage over eukaryotic hosts in terms of faster generation times and larger population sizes, and can thus generate mutants that exploit hosts without providing benefit in return (Herre *et al.* 1999; Sachs *et al.* 2004). Horizontal transmission of symbionts (i.e., from the environment and not directly from parent to offspring) requires bacterial symbionts to adapt to environments outside of hosts, uncoupling the fitness interests between mutualist partners (Frank 1996). Hence, not only are environmentally acquired mutualisms are predicted to be evolutionarily unstable (Keeler 1985; Bull & Rice 1991; Sachs 2006;

Sachs & Simms 2006, 2008), but variation in symbiotic quality could be driven by evolutionary instability within symbiotic traits. Symbiotic quality can also be context dependent; benefits provided can vary with external resources, biotic environment, combinations of host and symbiont genotype, in time and/or space, and with interactions among these factors (Bronstein 1994a). Symbiont quality is often studied mostly among beneficial genotypes, but we have little understanding of how context dependency shapes the evolution of *ineffective* symbionts. Moreover, variation in fitness benefits are typically studied only among mutualist partners, ignoring the potential competitive effects of environmental microbes. Thus, it is unclear if non-mutualistic conspecific partners can modulate mutualism benefits.

The legume-rhizobium mutualism is a key system to study variation in symbiont quality. Rhizobia are soil bacteria with diverse lifestyles (Denison & Kiers 2004). Through a complex signaling exchange, some lineages infect plant roots, form a plant-derived organ (nodule), differentiate, and fix nitrogen for the host in exchange for photosynthates (Sprent *et al.* 1987; Lodwig *et al.* 2003). Among nodulating rhizobia, nodulation and nitrogen fixation genes are typically encoded on symbiosis plasmids (Galibert *et al.* 2001; Young *et al.* 2006) or on genomic islands (i.e., the "symbiosis island") (Kaneko *et al.* 2000; Göttfert *et al.* 2001; Kaneko *et al.* 2002; Lee *et al.* 2008). Nodulating rhizobia vary naturally in symbiotic quality, ranging from highly beneficial wherein genotypes enhance host growth through substantial nitrogen fixation, to ineffective wherein rhizobia nodulate the host but do not provide growth benefits (Burdon *et al.* 1999; Simms *et al.* 2006; Sachs *et al.* 2010). These different nitrogen

fixing strategies (i.e., high vs low) represent two lifestyle alternatives for rhizobial bacteria. Yet, the benefits provided by nodulating rhizobia to legume hosts can be context dependent, varying with extrinsic environment (Regus *et al.* 2014; Simonsen & Stinchcombe 2014), host and symbiont genotype combination (Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath 2010), and with interactions between genotype and environment (Heath & Tiffin 2007). A third rhizobial lifestyle includes lineages that dominate soil populations (Jarvis *et al.* 1989; Segovia *et al.* 1991; Laguerre *et al.* 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996; Pongsilp *et al.* 2002; Sachs *et al.* 2009; VanInsberghe *et al.* 2015; Hollowell *et al.* 2016), but do not nodulate legume hosts, most likely because they lack critical loci needed for symbiosis (Sachs *et al.* 2010; Okubo *et al.* 2012). The differential fitness effects of beneficial rhizobia due to context dependency have been studied in the most depth, but the evolution and the context dependent effects of ineffective and non-nodulating rhizobia are unclear.

This dissertation examines drivers of symbiotic quality in the native *Acmispon-Bradyrhizobium* mutualism. *Acmispon strigosus* (formally *Lotus*) is an annual legume native to California that interacts with diverse *Bradyrhizobium spp.* that range from highly beneficial, to ineffective, to non-nodulating (Sachs *et al.* 2009; Sachs *et al.* 2010). Although ineffective *Bradyrhizobium* have been uncovered (Sachs *et al.* 2010; Ehinger *et al.* 2014), few studies have examined the incidence of ineffective *Bradyrhizobium* in natural populations or directly measured nitrogen fixation ability of symbionts. In the first chapter of my dissertation, I investigated the evolutionary stability and structure of symbiotic function across a metapopulation of *A. strigosus* hosts. I measured symbiotic

effectiveness and nitrogen fixation of eighty-five *Bradyrhizobium* isolates and reconstructed phylogenetic relationships using four loci.

In the second chapter, I examined the maintenance of ineffective rhizobia in a metapopulation of native hosts and symbionts that experience spatial structure in soil nitrogen availability. I quantified fitness and fitness effects of diverse *Bradyrhizobium* isolates on sympatric and allopatric *Acmispon* hosts and tested two contrasting frameworks that model the persistence of ineffective rhizobia; one that predicts ineffectiveness occurs as a maladapted, context dependent trait and the other that predicts ineffective rhizobia evolve adaptively to exploit host resources.

In the final chapter, I assessed the effects of non-nodulating *Bradyrhizobium* on the benefits provided during symbiosis. I performed clonal inoculations of diverse nodulating and non-nodulating isolates and also co-inoculated hosts with mixtures of nodulating and non-nodulating isolates. I tested if the presence of non-nodulating *Bradyrhizobium* could modulate the benefits provide to each partner during the mutualism.

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CHAPTER 1

Recurrent loss of nitrogen fixation function in natural *Bradyrhizobium* populations

Abstract

To maximize benefits and minimize costs of microbial mutualisms, eukaryotic hosts must selectively reward beneficial symbionts and punish ineffective genotypes. However, little is known about the impact of these host traits on symbiont populations. Here, we investigate variation in key symbiotic traits in *Bradyrhizobium spp.* that are root nodulating symbionts of the legume *Acmispon strigosus*. *A. strigosus* has been demonstrated to reward nitrogen-fixing *Bradyrhizobium* and to efficiently sanction ineffective genotypes. We measured nitrogen fixation function and host-growth effects of eighty-five *Bradyrhizobium* isolates collected from a natural metapopulation of *A. strigosus* hosts. We reconstructed phylogenetic relationships among *Bradyrhizobium* isolates using loci expressed both during symbiotic and free-living rhizobial phases and analyzed the evolution of symbiotic traits. We uncovered patterns consistent with rapid shifts in symbiotic effectiveness and repeated loss of nitrogen fixation function. Symbiotic effectiveness varied markedly within most populations but with little variation among them. These data suggest that forces beyond host selection are shaping symbiont populations. The drivers shaping symbiotic traits appear to act primarily within populations, and can include mutation-selection balance, selection for host exploitation, and selection in free-living phases between host infection.

Introduction

Bacterial mutualisms with eukaryotic hosts are intimate, reciprocally beneficial interactions that are ubiquitous across hosts and habitats (Douglas 2010). The bacterial partners in these interactions (i.e., symbionts) are dynamic players, positively shaping host development, physiology, pathogen defenses, and tolerance to stress and disease (Mueller & Sachs 2015). Eukaryotic hosts are predicted to be a dominant selective force shaping bacterial symbiont populations. To minimize the potential for symbiont exploitation, hosts must selectively favor symbiont cooperation and punish ineffective (nonbeneficial) symbiont genotypes. Models of 'host control' predict that hosts select for beneficial symbionts through specificity prior to infection (Simms & Taylor 2002; Sachs *et al.* 2004) and via sanctions of ineffective partners after infection (Denison 2000; West *et al.* 2002a; West *et al.* 2002b). Under host control models, beneficial symbiont genotypes are favored and ineffective genotypes are predicted to be extirpated from symbiont populations (Bull & Rice 1991; Denison 2000; West *et al.* 2002a; West *et al.* 2002b; Foster & Kokko 2006). However, scant empirical data exists on the frequencies of beneficial and ineffective symbiotic partners in natural populations. Despite the wealth of theory on host-symbiont interactions, we have relatively little understanding of how key symbiont traits evolve, and whether and when these functions respond to host selection.

The legume-rhizobium mutualism is an ecologically and economically important symbiosis and is an ideal system to study the evolution and variation of symbiotic traits. Rhizobial bacteria form nodules on legume roots and fix nitrogen for the host in exchange for photosynthates (Sprent *et al.* 1987; Lodwig *et al.* 2003). Legumes can

discriminate against ineffective genotypes subsequent to nodule development – and such legume sanctions are thought to be the primary mechanism that constrains rhizobial exploitation (Singleton & Stockinger 1983; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010a; Oono *et al.* 2011; Regus *et al.* 2014). Under legume sanctions, nodules containing beneficial rhizobia grow rapidly (and rhizobia within them proliferate), whereas nodules with ineffective rhizobia stay small (and the rhizobia within them have reduced population sizes) (Singleton & Stockinger 1983; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010a; Oono *et al.* 2011; Regus *et al.* 2014). Similar to other symbioses such as between plants and mycorrhizae (Johnson *et al.* 1997), plants and *Frankia* (Markham 2008), and squid and *Vibrio* (Nishiguchi & Nair 2003), there can be substantial variation in rhizobial symbiotic quality (Quigley *et al.* 1997; Burdon *et al.* 1999; Denton *et al.* 2000; Chen *et al.* 2002; Collins *et al.* 2002; Sachs & Simms 2006; Sachs *et al.* 2010b). However, rhizobial effectiveness has rarely been examined in a phylogenetic context and thus its evolution is poorly understood. Moreover, little is known about the origins of ineffective rhizobia despite their potential role in destabilizing this mutualism (Sachs & Simms 2008).

Here, we examined nitrogen fixation function, host fitness effects, and rhizobial fitness of eighty-five *Bradyrhizobium* symbionts isolated from a natural metapopulation of *Acmispon strigosus* hosts. *A. strigosus* (formally *Lotus strigosus*) is an annual legume native to the Southwestern USA that is nodulated by diverse *Bradyrhizobium spp*. (Sachs *et al.* 2009). Previous work focusing on one population of *A. strigosus* revealed extensive variation in *Bradyrhizobium* effectiveness on the sympatric hosts; *Bradyrhizobium*

isolates ranged from highly beneficial, wherein genotypes enhanced host growth through substantial nitrogen fixation, to symbiotically ineffective wherein genotypes nodulated the host but did not enhance host growth due to little to no nitrogen fixation (Sachs *et al.* 2010b). *A. strigosus* has been shown to efficiently express sanctions traits in greenhouse experiments (Sachs *et al.* 2010a; Regus *et al.* 2014). Studies of *A. strigosus* sanctions traits reveal that ineffective *Bradyrhizobium* are efficiently sanctioned irrespective of variation in light regime, or nitrogen inputs, suggesting that *A. strigosus* sanctions should be robust to ecological variation (Regus *et al.* 2014; Regus *et al.* 2015). In the present experiments we examined symbiotic effectiveness and fitness traits of diverse *Bradyrhizobium* isolates. We reconstructed phylogenetic relationships among the *Bradyrhizobium* isolates and used the tree to test hypotheses about the evolution of symbiotic effectiveness. Three main questions were investigated: i) To what degree does nitrogen fixation function vary within and among closely related lineages of *Bradyrhizobium*? ii) How often are ineffective *Bradyrhizobium* isolates uncovered and given the potential to exploit hosts, is there evidence for the local spread, fixation, or diversification of ineffective rhizobia? and iii) How is nitrogen fixation function structured within and among host populations?

Materials and Methods

Bradyrhizobium isolates

A collection of 1292 *Bradyrhizobium spp.* isolates were previously cultured from nodules and the root-soil interface of *Acmispon spp.* (Sachs *et al.* 2009; Hollowell *et al.* 2016a; Hollowell *et al.* 2016b) and from bulk soil adjacent to *A. strigosus* hosts at thirteen natural sites across California. Bulk soil isolates were collected from four Southern California *A. strigosus* populations (University of California Riverside, Robert J. Bernard Biological Field Station of the Claremont Colleges, Anza Borrego Desert State Park, and Burns Piñon Ridge Reserve near Yucca Valley). Soil cores were collected in August 2014, sieved to 2mm, saturated with sterile water, filtered through 8 layers of cheesecloth, and the resultant supernatant was inoculated onto axenic *A. strigosus* seedlings from originating from sympatric sites (August 14th 2014). Plants were raised 6 weeks in a growth room, fertilized weekly with nitrogen-free Jensen's (Somasegaran & Hoben 2012), de-potted and washed to examine roots. Bulk soil isolates were only cultured from white or yellow nodules (i.e., that are lacking leghemoglobin associated with symbiotic nitrogen fixation) to improve chances of isolating ineffective rhizobial genotypes. Nodules were removed, stored at 4°C (1-14 days), cultured onto MAG plates, and a single colony per nodule was grown and archived. Isolates from ten collection sites were analyzed in this study (**Table 1.1**).

Bradyrhizobium genotype database and selection of isolates for analysis

Bradyrhizobium isolates were previously sequenced at two loci located on the bacterial chromosome (i.e., CHR: *glnII*, *recA*) and were assigned to chromosomal genotypes (Hollowell *et al.* 2016a). A subset of these isolates (collected from nine of sites; 358 isolates) were previously sequenced at two loci located on the *Bradyrhizobium* symbiosis island (*nodZ*, *nolL*), a large genomic island that encodes symbiotic functions, and were assigned to symbiosis island genotypes based on these loci (i.e., SI; (Hollowell *et al.* 2016b)). Using the above sequence databases, we estimated local genotype abundance for each sequenced genotype, defined as the proportion of the total inferred nodulating isolates in an individual population (i.e., field site) encompassed by the genotype. Chromosomal abundance was estimated using the CHR dataset (Hollowell *et al.* 2016a) and SI abundance was estimated using the subset of data that included SI loci (Hollowell *et al.* 2016b). Comparison of relative proportional genotype abundance can serve as a proxy of genotype fitness within a population.

Eighty-five isolates were chosen for analysis in this study. Isolates were collected from ten *A. strigosus* populations, not all of which had complete SI sequence data, across California that have been shown to exhibit population structure in their *Bradyrhizobium* communities (Hollowell *et al.* 2016a; Hollowell *et al.* 2016b) and experience ~10× variation in soil nitrogen concentration (Fenn *et al.* 2010; Regus *et al.* 2014). Sixty-two isolates from nodules, eight from the root surface, and fifteen from bulk soil were selected. An average of eight isolates were selected per field site (range: ±5 isolates, SE ± 1.58) (**Table 1.1**). All selected isolates except those from bulk soil were previously

assigned CHR genotypes (Hollowell *et al.* 2016a) and most were assigned SI genotypes (Hollowell *et al.* 2016b) (**Table 1.1**). From the sequenced isolates, we attempted to include the broadest range of genotype abundance values for the CHR from each population. The bulk soil isolates were sequenced for this study at the CHR and SI loci using published protocols and were also assigned to genotypes as described above (Stępkowski *et al.* 2005; Vinuesa *et al.* 2008; Hollowell *et al.* 2016a; Hollowell *et al.* 2016b). For nodule and root surface isolates with missing SI data (**Table 1.1**), symbiosis loci were sequenced and assigned to SI genotypes (Stępkowski *et al.* 2005; Hollowell *et al.* 2016a; Hollowell *et al.* 2016b). Missing sequence data was add to the CHR and SI datasets and local genotype abundance was then estimated for each isolate included in this study.

Inoculation experiments

Bradyrhizobium isolates were grown from original frozen stocks and streaked onto plates with Modified Arabinose Gluconate medium (MAG) (Sachs *et al.* 2009) and incubated until lawns formed (29°C, ~8 days). Rhizobial cells were washed from plates and resuspended in liquid MAG to estimate concentrations via optical density (Sachs *et al.* 2010b). Resuspended cells were then centrifuged (4000 rpm, 20 minutes) to remove media and resuspended again in sterile water at 10^8 cells ml^{-1}. Inoculated plants received 5×10^8 rhizobial cells in 5ml of sterile water and uninoculated controls received 5ml of sterile water.

A. strigosus is a permissive host that forms nodules with diverse *Bradyrhizobium spp.* (Sachs *et al.* 2009; Hollowell *et al.* 2016a; Hollowell *et al.* 2016b). Rhizobial symbiotic effectiveness can vary depending on rhizobial genotype, host genotype, and their interaction (i.e., G×G interactions; (Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath 2010)). A subset of isolates included in this study were previously examined on multiple host genotypes and G×G interactions were found to be negligible (Wendlandt 2017 *under review*, Chapter 2 of this dissertation). Thus, a single inbred *A. strigosus* host line was used in this inoculation experiment (AcS049.Cla.m01.g1.r02). *A. strigosus* seeds were surface sterilized, nick scarified, and germinated in sterile nitrogen-free Jensen's solution (Somasegaran & Hoben 2012). Seedlings were planted into sterilized conetainers (Steuwe and Sons) filled with sterilized quartzite sand, incubated in a growth chamber for two weeks, and moved to the greenhouse under ~50% shade for hardening (4 days, 1 × daily misting) (Sachs *et al.* 2009). One week after planting, seedlings were fertilized with 1ml of sterile nitrogen-free Jensen's solution, which was increased to 3ml per plant at two weeks after planting. Beginning three weeks after planting (~2 days before inoculation), plants were fertilized weekly with 4.5ml Jensen's solution supplemented with a low concentration of dissolved potassium nitrate (KNO_3; 0.05 g L^{-1}; 5% atm^{15}N). This fertilization treatment was used to optimize estimation of atm%^{15}N in grown plants and represents ~10% of the KNO_3 concentration needed to maximize *A. strigosus* shoot growth in the absence of rhizobial infection (Regus *et al.* 2014; Regus *et al.* 2015).

Axenic seedlings were arranged by size and groups of size matched seedlings were randomly assigned to inoculation treatments and divided into blocks accordingly.

Bacterial treatments were separated into four inoculation groups and inoculated on separate days (March 27th-29th and April 3rd 2016), wherein each group included an uninoculated control treatment (**Table 1.1**). All plants within a treatment were inoculated on the same day. Each treatment was replicated on ten plants separated into individual blocks, except for treatments in the last inoculation group which had five replicate plants divided into separate, individual blocks (89 treatments × 1 host line × 10 replicates per treatment, except for inoculation group 4 which had 5 replicates = 805 plants total). Plants were harvested approximately eight weeks after inoculation in the same order as treatment inoculation. During harvest, four replicate plants per treatment in inoculation groups 1-3 and all replicate plants for inoculation group 4 were removed from the pots and soil was separated from the roots by washing with tap water (Inoculation groups 1-3: May 19th-24th 2016, Inoculation group 4: May 31st-June 3rd 2016). For any treatments in which replicate plants exhibited inconsistent nodulation or the absence of nodules, all plant replicates were de-potted and washed to inspect for nodules. For the remaining plant replicates in each treatment shoots were removed and dried and roots were disposed. Washed plants with roots were individually wrapped and stored on trays at 4°C until dissection (Inoculation groups 1-3: May 19th-June 3rd 2016, Inoculation group 4: June 3rd-24th 2016). During plant dissections, nodules were removed from the roots, counted, and photographed. Roots, shoots, and nodules were separated and oven dried (60°C, >4 days) prior to weighing dry biomass.

Leaf atm%^{15}N assays

We measured leaf atom percent ^{15}N (atm%^{15}N) and analyzed differences between inoculated and sized matched uninfected plants for each *Bradyrhizobium* isolate. Rhizobia preferentially fix ^{14}N due to isotopic fractionation, thus when plants incorporate symbiotically fixed nitrogen, plant leaves exhibit lower atm%^{15}N relative to uninfected plants (Yoneyama *et al.* 1986). Leaflets from four replicates per treatment were removed from dried shoots and ground to a fine powder for analysis. Tissues were analyzed at the University of California Santa Cruz stable isotope laboratory.

Phenotypic classification of *Bradyrhizobium* isolates

Isolates were categorized as non-nodulating if they failed to form nodules on all inoculated hosts. Alternatively, isolates were categorized as nodulating if they successfully formed nodules on all inoculated hosts. Inoculation treatments that resulted in inconsistent nodulation of all inoculated hosts were considered to have a mixed nodulation phenotype. For treatments with consistent nodulation, symbiotic effectiveness was estimated as the host's growth response to *Bradyrhizobium* inoculation relative to size-matched uninoculated controls (HGR = [(Shoot mass Inoculated Plant – Shoot mass Control Plant)/Shoot mass of Control Plant] × 100; (Sachs *et al.* 2010b)). In past research *Bradyrhizobium* isolates have been classified as ineffective if they consistently formed nodules but did not cause significant host growth compared to uninoculated controls or if there were no differences in atm%^{15}N between inoculated plants and uninoculated controls. Here we combined these metrics by using a principle components analysis

(PCA) with a clustering algorithm to categorize isolates as effective or ineffective (**Figure S1.1**). Principle components, clustering, and correlation analyses were calculated with isolate means for each trait and were log transformed to improve normality in JMP v 10.0 (Inc. 2012).

Phylogenetic reconstructions and trait analyses

Bayesian phylogenetic trees were reconstructed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) with the following parameters: GTR + I + G model of evolution (Sachs *et al.* 2011b), 5×10^6 generations, a heating temperature of 0.01, a 'burnin' of 12,000 trees, and two parallel runs starting with random trees, each with four simultaneous chains. Concatenated sequences of the *glnII*, *recA*, *nodZ*, *nolL* loci were aligned using Clustal Omega (Sievers *et al.* 2011) with default parameters and gaps were treated as missing data. MAFF303099 was used as an outgroup. A plot of log-likelihood scores of sampling points (sample frequency = 500) against generation number was observed in each case to ensure that stationarity had been reached during the 'burnin' period. Because the symbiosis island can be horizontally transferred among rhizobial lineages (Sullivan & Ronson 1998), phylogenies were also reconstructed separately for the CHR (**Figure S1.2**) and SI loci (**Figure S1.3**) using the same parameters except with a 'burnin' of 10,000 trees.

For each trait that we measured (host growth response, atm%^{15}N, mean individual nodule mass, and local CHR and SI abundance) we used Bayesian phylogenies reconstructed with the four locus dataset to test for phylogenetic signal (the tendency of

more closely related taxa to resemble one another compared to more distantly related taxa). In cases where a genotype included multiple isolates, a representative isolate was randomly selected to include in analyses. This approach eliminates polytomies. Phylogenetic signal was estimated using Blomberg's *K* for continuous variables (including host growth response, atm%^{15}N, mean individual nodule biomass, local CHR abundance, and local SI abundance) using the "phylosignal" function in the "picante" R package (Kembel *et al.* 2010), where *K* compares the observed signal in a trait to the signal under a Brownian motion (BM) model (Blomberg *et al.* 2003). *K* values close to 1 indicate a BM process and suggests some degree of phylogenetic signal, whereas *K* values close to 0 correspond to a random pattern of trait evolution. We tested if *K* was significantly greater than 0 (i.e., no phylogenetic signal) with 999 randomizations and report the mean ±SE of *K* and average *P*-values calculated across 20 trees to account for phylogenetic uncertainty (Koski & Ashman 2016). We also tested the separate CHR and SI consensus trees for phylogenic signal using Blomberg's *K*. If a trait did not exhibit phylogenetic signal, data gathered from each *Bradyrhizobium* isolate was treated independently. For the binary trait of nodulation ability (nodulating = 1, non-nodulating = 0), we used a Bayesian phylogeny reconstructed with the CHR dataset that included additional phenotyped isolates (Sachs *et al.* 2009; Sachs *et al.* 2011b; Hollowell *et al.* 2016a) and tested for phylogenetic signal using Pagel's lambda with the "fitDiscrete" function in the "geiger" R package (Pagel 1999; Harmon *et al.* 2008). A lambda value near 0 indicates that the tree topology does not structure trait variation (i.e., phylogenetic signal), as opposed to a lambda value near 1, which suggests that the trait is distributed

on the tree in accordance with BM. We tested if lambda was significantly > 0 by comparing the log-likelihood of the fitted lambda with that of lambda = 0 using a log-likelihood ratio test (Koski & Ashman 2016).

Data Analysis

We used nodule number and mass as *in planta* proxies for rhizobial fitness, as both of these parameters are positively correlated with rhizobial populations sizes in *A. strigosus* (Sachs *et al.* 2010b) and in other systems (*Medicago truncatula*: (Heath & Tiffin 2007, 2009); *Glycine max:* (Kiers *et al.* 2003); *Lupinus arboreus:* (Simms *et al.* 2006)). Variation in symbiotic effectiveness (i.e., HGR, atm%^{15}N) and rhizobial fitness (i.e., nodule number, mean individual nodule mass) among isolates and within each collection site was analyzed using ANOVAs. Generalized linear mixed models (GLMMs) were used to analyze variation among collection sites (fixed effect: collection site, random effect: isolate). All response variables were log transformed to improve normality when necessary and ANOVAs or GLMMs with significant *F* ratio statistics were followed by Tukey's HSD test to test for differences among treatments (Inc. 2012).

Results

Genotypic variation in symbiotic traits

Seventy-nine of the eighty-five *Bradyrhizobium* isolates formed nodules on all inoculated plants. Of the remaining isolates, five failed to nodulate any hosts (#'s 40, 44, 53, 61, and 199) and #149 formed a single nodule on one plant replicate (**Table 1.2**). Isolate #199 was originally cultured from an *A. strigosus* nodule, suggesting that it might have coinfected the original host with a nodulating strain (i.e., (Rangin *et al.* 2008; Gano-Cohen *et al.* 2016)). Moreover, #199 assimilated symbiotically fixed nitrogen (atm%^{15}N was significantly decreased compared to uninfected controls), but did not increase host growth suggesting that it could be adapted as a non-nodule forming endophyte. Isolate #'s 44 and 61 significantly increased *A. strigosus* host growth, but did not substantially assimilate symbiotically fixed nitrogen (**Table 1.2)**. None of the uninoculated control plants formed nodules. Inoculation treatments that did not consistently form nodules and uninoculated controls were removed from remaining analyses.

All effective *Bradyrhizobium* isolates clustered together and principle component 1 (i.e., atm%^{15}N) explained ~88% of the variation (**Figure 1.1**). Six ineffective isolates were recovered from five collection sites (**Table 1.3**; **Figure 1.1, Figure 1.3**). Host growth response and -atm%^{15}N were positively correlated (R^2 = 0.662, P < 0.0001, n=79), consistent with substantial plant assimilation of symbiotically fixed nitrogen. Host growth response and mean individual nodule mass were also positively correlated (R^2 = 0.209, P < 0.0001, n=79), suggesting that *A. strigosus* hosts preferentially reward more beneficial isolates *in planta* among the single inoculation treatments.

Phylogenetic Analyses

We were unable to successfully sequence symbiosis island loci for non-nodulating isolates, consistent with previous work (Sachs *et al.* 2011b; Hollowell *et al.* 2016a). Moreover, for *nodZ* there were four nodulating isolates (#'s 170, 189, 190, 200) that we could not sequence and one isolate for *nolL* (#182). The *Bradyrhizobium* phylogeny is mostly well resolved, with 26 clades supported by posterior probabilities ≥ 0.80 (**Figure 1.2**). Non-nodulating genotypes were descendant from four distinct clades and two non-nodulating isolates were found as sister taxa to ineffective genotypes. Ineffective isolates also did not compose a monophyletic group, and instead were independently found in three well supported clades (pp ≥ 0.80) and in two poorly supported lineages (pp ≥ 0.50) (**Figure 1.2**). Similar results were found on the separate CHR and SI phylogenies (**Figures S1.2** and **S1.3**, respectively). We did not observe significant phylogenetic signal for host growth response, atm%^{15}N, mean individual nodule mass, and local CHR or SI abundance (**Table 1.4; Figure 1.2**).

Variation in symbiotic traits

Trait data were treated as independent of the phylogeny and analyzed using GLMMs and ANOVAs. We found mixed evidence for local fixation of beneficial rhizobial genotypes within symbiont populations; at least one ineffective isolate was recovered from five collection sites and the remaining 5 sites only contained isolates that provided hosts with significant fixed nitrogen and significant fitness benefits (**Figure 1.3**). Four (of 6) ineffective isolates exhibited low abundance within their respective

populations (≤ 0.015 at both the CHR and SI loci except for #187 for the CHR and #200 for the SI) (**Figure 1.2, Table 1.1**). Isolates CW1 and #155 had relatively high abundance, but neither genotype spread to fixation within their respective populations (collected from UCR: abundance ≥0.375 and YUC: abundance ≥0.225, respectively) (**Figure 1.2, Figure 1.3, Table 1.1**).

We uncovered significant variation among isolates in most populations (**Table S1.1**). For host growth response and atm%^{15}N, no significant variation among isolates was uncovered for ANZ, GP, and MOT. Isolates collected from WHT also did not differ in the amount of symbiotically fixed nitrogen (**Table S1.1**). No significant variation in nodule number among isolates was found at CLA, GP, MOT, SAN, UCR, WHT, or YUC (**Table S1.1**). Isolates from GP, MOT, WHT, and YUC did not exhibit variation in mean individual nodule biomass (**Table S1.1**).

Nitrogen fixation did not vary significantly among populations (**Figure 1.3a** and **b** and **Table 1.5**). For host growth response only the GP and PIS populations could be statistically differentiated from each other. For nodule number, only MOT and SAN were distinct from the ANZ population (**Figure 1.3c** and **d; Table 1.5**). For mean individual nodule mass CLA and ANZ were the only populations significantly different from PIS.

Discussion

Eukaryotic hosts most often infectiously acquire beneficial symbionts from the environment anew each host generation (i.e., horizontal transmission), as opposed to directly from their parents (i.e., vertical transmission) (Sachs *et al.* 2011a). Despite mechanisms that can select for mutualism stability (reviewed in (Bronstein 1994b; Sachs *et al.* 2004)), two key forces can disfavor cooperation in horizontally transmitted mutualisms. First, bacterial symbionts have a substantial evolutionary advantage over eukaryotic hosts, in terms of generation times and population sizes, and can rapidly generate mutants that exploit hosts without providing benefit in return (Herre *et al.* 1999; Sachs *et al.* 2004). Second, horizontal transmission requires bacterial partners to adapt to environments outside of hosts, potentially favoring a set of traits that can counteract symbiotic effectiveness (Frank 1996). Due to these selective forces, environmentally acquired mutualisms are often predicted to be evolutionarily unstable (Keeler 1985; Bull & Rice 1991; Sachs 2006; Sachs & Simms 2006, 2008). Empirical work had uncovered evidence of symbiosis instability in terms of the recurrent loss the capacity to interact with hosts (Hibbett *et al.* 2000; Wilkinson & Sherratt 2001; Nishiguchi & Nair 2003; Sachs & Simms 2006; Sachs *et al.* 2009; Sachs *et al.* 2010b; Sachs *et al.* 2011b; Sachs *et al.* 2011c). For example, several lineages of mycorrhizal symbionts have been shown to exhibit evolutionary reversals to saprotrophy. Hibbett and colleagues (2000) demonstrated at least nine evolutionary reversals from ectomycorrhizal symbiosis to saprotrophic growth in the soil. However, other than the wholesale loss of the capacity to interact with hosts, few studies have examined the fine scale variation in symbiotic

function over evolutionary time among host associated lineages (but see (Sachs *et al.* 2011b; Gordon *et al.* 2016)).

Legumes can efficiently select for beneficial rhizobia *in planta (Singleton & Stockinger 1983; Kiers et al. 2003; Simms et al. 2006; Sachs et al. 2010a; Oono et al. 2011; Regus et al. 2014)*, but rhizobia retain extensive environmental phases in the absence of host selection (Sprent *et al.* 1987; Denison & Kiers 2004). Selection for symbiotic traits by hosts is thus limited by rhizobial replication *in planta (Sachs & Simms 2006)*, whereas selection during environmental phases can promote traits for persistence within the soil at a cost to symbiotic function (Sachs & Simms 2008). For example, when multiple *Bradyrhizobium* genotypes were experimentally evolved *in vitro* for ~500 generations (i.e., without host interaction) this resulted in rapidly degraded symbiotic function, suggesting that host plants must continually select upon rhizobial effectiveness for it to be maintained (Sachs *et al.* 2011b). Similarly, a recent study investigated the evolution of nitrogen fixation function in *Rhizobium leguminosarum*, wherein rhizobia were exposed to selection in the presence and absence of nitrogen fertilization in a field experiment (Gordon *et al.* 2016). Phylogenic reconstruction of these isolates showed evidence for the evolutionary instability in nitrogen fixation function. Consistent with these studies, we recovered phylogenetic patterns in accordance with the repeated evolution of symbiotic ineffectiveness in host associated *Bradyrhizobium* across a metapopulation of hosts, suggesting that evolutionary loss of nitrogen fixation function might be a common process. Ineffective isolates in this study could not be resolved into a

single, independent monophyletic group, inconsistent with the spread and diversification of specific ineffective lineages (**Figure 1.2**).

Our data suggest that nitrogen fixation function is rapidly evolving in these natural isolates of *Bradyrhizobium*. Even among closely related genotypes, symbiotic quality often varied dramatically. For instance, most ineffective isolates were sister taxa to highly effective isolates (except #2) (**Figure 1.2**) Moreover, we did not uncover significant phylogenetic signal for host growth response or atm%^{15}N (**Figure 1.2; Table 1.4**), consistent with rapid evolution of nitrogen fixation ability. However, phylogenetic signal may not be detected for several other reasons. First, phylogenetic signal could be masked by poor phylogenetic resolution at the tips of the tree (Münkemüller *et al.* 2012). To examine this possibility, we added additional phenotyped isolates (Sachs *et al.* 2009; Sachs *et al.* 2011b; Hollowell *et al.* 2016a) to the CHR phylogeny to improve phylogenetic resolution and used Pagel's lambda to test a binary trait that was previously found to have phylogenetic signal (nodulation ability: (Hollowell *et al.* 2016a)) (**Figure S1.4**). Nodulation ability exhibited significant phylogenetic signal (lambda = 0.9497, $P = 8.8 \times 10^{-5}$), suggesting that the reconstructed phylogenies are well-resolved enough to detect phylogenetic signal. Second, tests for phylogenetic signal could have been confounded by horizontal gene transfer of the symbiosis island loci. To explore this potential, we examined phylogenetic signal separately on the CHR and SI phylogenies (**Table S1.2**). Although some traits exhibited K values significantly different from zero, phylogenetic signal estimates were negligible ($K < 0.08$, except for atm%^{15}N for the SI) (**Table S1.2**) suggesting that phylogenetic signal was not obscured due to concatenating

the CHR and SI loci. Third, phylogenetic signal could have been masked by inherent noisiness of the measured traits, wherein the amount of nitrogen fixed by a particular isolate can vary with host genotype and/or environmental conditions (i.e., G×G, G×E, and G×G×E interactions; (Bronstein 1994a; Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath 2010; Lau *et al.* 2012; Heath & Stinchcombe 2014)). However, symbiotic traits of *Bradyrhizobium* isolates tested here (host growth response and ^{15}N) have been shown to be consistent among studies, wherein effective and ineffective isolates are consistently beneficial or nonbeneficial, respectively (Sachs *et al.* 2010a; Sachs *et al.* 2010b; Regus *et al.* 2014; Regus *et al.* 2015; Gano-Cohen *et al.* 2016; Hollowell *et al.* 2016a, Wendlandt 2017 *in review*, Chapter 2 of this dissertation). Finally, phylogenetic signal could be lacking because of rapid evolution of *Bradyrhizobium* isolates. In contrast to a recent study, which found phylogenetic signal for symbiotic quality using the nitrogen fixation gene *nifD* in *Rhizobium leguminosarum* (Gordon 2016), we did not include analyses of genes directly involved in nitrogen fixation. Additional sequence analyses of genes directly involved in nitrogen fixation are necessary to determine if this is the case in *Bradyrhizobium* isolates. Nonetheless, the repeated recovery of ineffective isolates at the tips of the phylogeny (**Figure 1.2**), the relatively good resolution of our phylogenies, and the stability in expression and measurement of symbiotic traits, suggest rapid evolution is the most likely explanation for the observed lack of phylogenic signal.

We recovered very broad variation in symbiotic traits within each population, but little variation among populations. For any given trait, most isolates within a population

significantly differed from each other, but among populations only two or three populations (of ten) were able to be differentiated from the others (**Figure 1.3; Table S1.1**). The mechanisms that contribute to variation in symbiotic traits may differ within and among populations. Among populations, symbiotic traits have been predicted to vary due to resource availability, spatiotemporal differences in host control, and host-by-rhizobial genotype interactions. For example, the *Bradyrhizobium* populations sampled here span a soil nitrogen gradient (Fenn *et al.* 2010; Regus *et al.* 2014). In soils where nutrients are abundant, plants are predicted to switch to mineral nutrient sources (Bronstein 1994a; West *et al.* 2002a; Thrall *et al.* 2007; Shantz *et al.* 2016), downregulating sanctions and thus relaxing *in planta* selection on symbionts (Kiers *et al.* 2006; Kiers *et al.* 2007). Legume hosts can also vary spatiotemporally in sanctioning ability due to the local frequency of beneficial rhizobial partners and coevolution with symbionts (Foster & Kokko 2006; Steidinger & Bever 2014, 2016), thereby generating differences in rhizobial quality among populations. Expression of symbiotic traits can also be host dependent, wherein symbiotic function varies with host genotypes among populations (i.e., G×G interactions; (Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath 2010)). We uncovered little variation among host populations, suggesting that the processes that shape *Bradyrhizobium* nitrogen fixation function might be primarily driven within host populations.

Half of the assayed populations included both ineffective and beneficial genotypes, suggesting that even with relatively sparse sampling (~8 isolates assayed per population) there was little evidence of the fixation of beneficial symbionts. These data

suggest that there are other forces beyond host selection that can shape variation in symbiont populations. For instance, under selection-mutation balance, mutation events regularly generate low-quality or non-nitrogen fixing rhizobia that are slowly purged from the population by negative selection (Van Dyken *et al.* 2011). Four (of six) ineffective isolates uncovered in this study are relative rare (**Figure 1.2**, **Table 1.1**) and it is possible that we captured ineffective mutants prior to their extirpation. There was also a positive relationship between symbiotic effectiveness (measured by HGR) and rhizobial fitness (measured by individual nodule biomass). This suggests *A. strigosus* preferentially rewards more beneficial isolates and is consistent with fitness alignment between partners (Friesen 2012). Thus, the low abundance of most ineffective isolates suggests selection-mutation balance could maintain variation in symbiont quality, at least within some populations. Alternatively, sanction traits among legume hosts could vary such that they are not capable of expressing sanctions until ineffective genotypes increase within the population (Steidinger & Bever 2014, 2016). Herein ineffective genotypes could persist and replicate in the soil in the absence of host sanctions, steadily increasing their frequency within the population. Data from our most well studied population (BMR) is inconsistent with this hypothesis. *Acmispon strigosus* hosts from BMR exhibit very effective host sanctions, and although an ineffective isolate has been uncovered from this population, it is still relatively rare (**Figure 1.2**) (Sachs *et al.* 2009; Sachs *et al.* 2010a; Sachs *et al.* 2010b; Sachs *et al.* 2011b; Regus *et al.* 2014; Regus *et al.* 2015). Rare ineffective genotypes could also found a nodule, but escape sanctions within a mixed nodule infection (Kiers *et al.* 2006; Kiers *et al.* 2013). Ineffective genotypes that found a

nodule would thus gain a substantial fitness benefit compared to conspecifics within the soil (Denison & Kiers 2004), but would not be immediately punished by host sanctions. The relatively high abundance of isolates CW1 and #155 (**Figure 1.2**) could potentially be explained by either of these mechanisms. However, once ineffective genotypes reach high enough frequency, sanctions are predicted to select against ineffective symbionts removing them from symbiont populations. Any of these three models, or combination thereof, could maintain variation in symbiotic function within populations.

We found striking evidence for the evolutionary instability of symbiotic quality within a metapopulation of *Bradyrhizobium*. We repeatedly uncovered ineffective isolates that could not be resolved into a single, independent monophyletic group. Moreover, we did not uncover significant phylogenetic signal for two metrics of symbiont quality (i.e., host growth response and amount of symbiotically fixed nitrogen). Combined, these data suggest that mutants that lose nitrogen fixation function, can persist natural rhizobial populations, and occasionally achieve significant local abundance. Nitrogen fixation function was structured within, but not among host populations, suggesting there may be different mechanisms that contribute to variation in symbiotic traits. Future work more closely examining genotype abundance and specific nitrogen fixation genes may reveal specific mechanisms that maintain variation in symbiotic function within populations.

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Figure 1.1 Classification of *Bradyrhizobium* isolates. Principle components and clustering analyses with mean atm%^{15}N, mean host growth response, and weight percent nitrogen for each isolate.

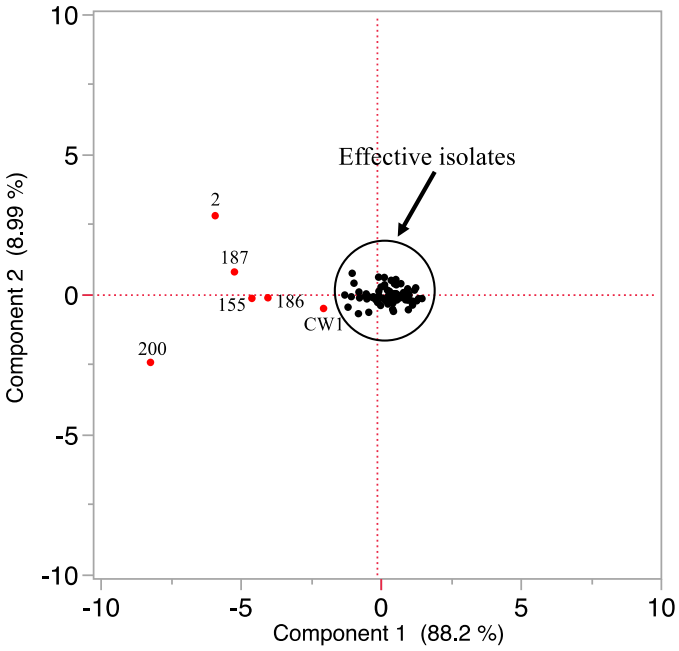
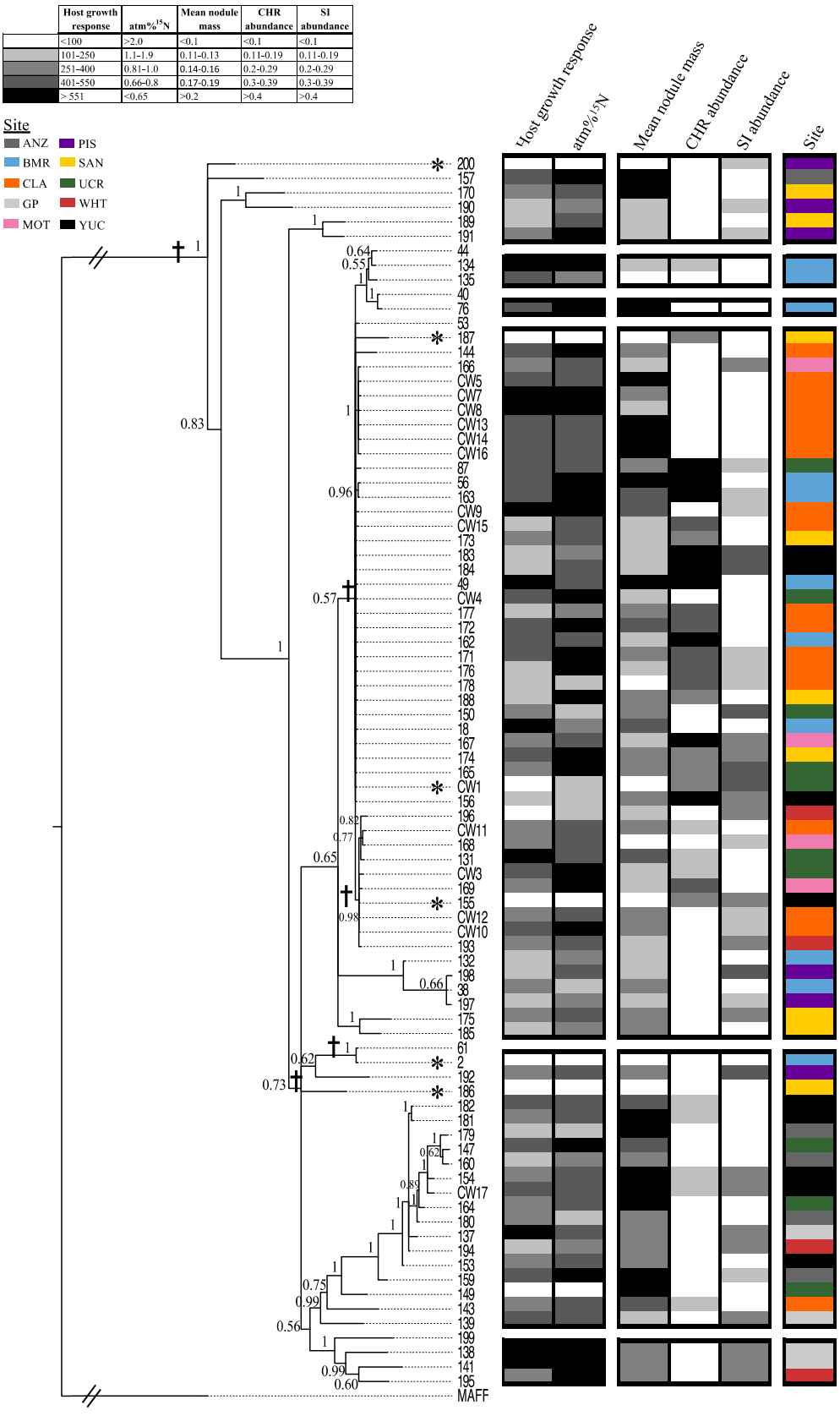


Figure 1.2 Phylogenetic signal on reconstructed *Bradyrhizobium* phylogeny. Bayesian phylogram rooted with MAFF of 85 inoculated *Bradyrhizobium* isolates reconstructed from concatenated *glnII*, *recA*, *nodZ*, *nolL* with corresponding heat map of continuous traits. Crosses indicate nodes that include ineffective isolates, which are identified with asterisks. Hashes indicate long branches shortened for visibility. The tree represents a single sample from the post-burnin set of trees, in which branch lengths are scaled to indicate number of nucleotide changes. Bayesian clade support values (posterior probabilities) are reported above the branches.



	Host growth response	atm%^{15}N	Mean nodule mass	CHR abundance	SI abundance
	<100	>2.0	<0.1	<0.1	<0.1
	101-250	1.1-1.9	0.11-0.13	0.11-0.19	0.11-0.19
	251-400	0.81-1.0	0.14-0.16	0.2-0.29	0.2-0.29
	401-550	0.66-0.8	0.17-0.19	0.3-0.39	0.3-0.39
	> 551	<0.65	>0.2	>0.4	>0.4

Figure 1.3 Variation in symbiotic traits. (a) Host growth response calculated using all inoculated plants (b) atm%^{15}N, (c) Nodule number, and (d) mean individual nodule mass calculated using the subset of plants for which below ground traits were collected (i.e., ~4-5 replicates per treatment). Colors indicate different rhizobial collection sites and asterisks identify ineffective isolates. Significant differences among collection sites are indicated with capital letters (see **Table S1.1** for differences among isolates within a collection site). Error bars represent 1 standard error.

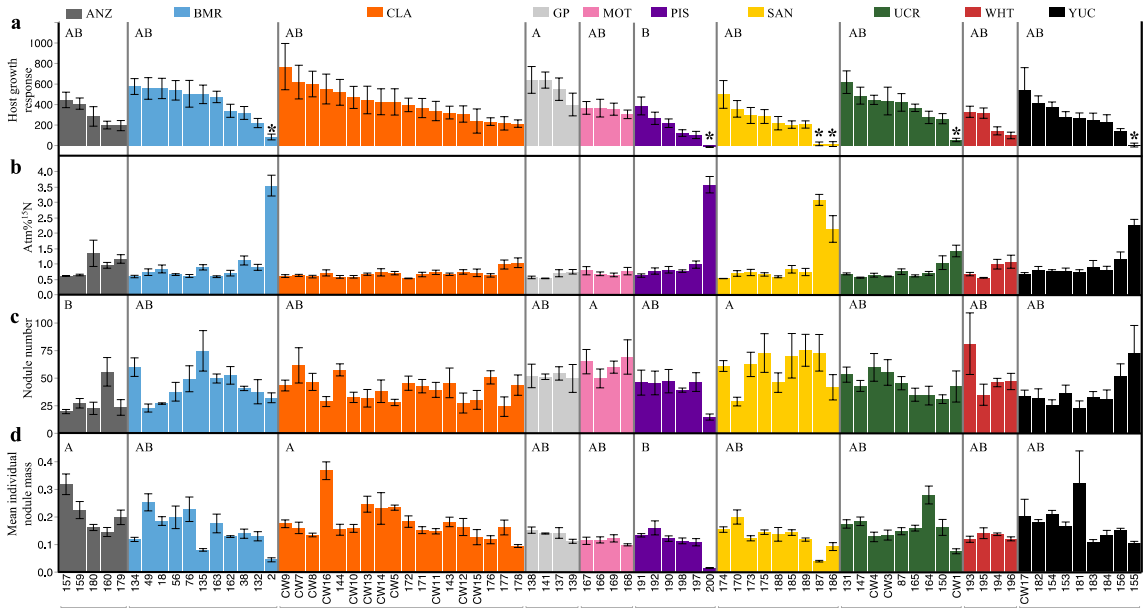


Table 1.1 Summary of *Bradyrhizobium* isolate information

Inoc group	Inoc #	Isolate	Site[#]	*glnII_recA* genotype	CHR abund[‡]	*nodZ_nolL* genotype	SI abund	Full genome genotype
1 - March 27th 2016								
	2	05LoS24R3_28	BMR	G14_R14	0.007	Z59_L74*	0.009	G14_R14_Z59_L74
	18	05LoS21R6_43	BMR	G117_R01	0.007	Z02_L04*	0.064	G117_R01_Z02_L04
	38	05LoS3_3	BMR	G106_R04	0.014	Z30_L39	0.009	G106_R04_Z30_L39
	40[+]	05LoS16R3_25	BMR	G08_R02	N/A	N/A	N/A	G08_R02_X_X
	44[+]	05LoS16R2_18	BMR	G07_R02	N/A	N/A	N/A	G07_R02_X_X
	49	05LoS23R7_12	BMR	G03_R01	0.518	Z02_L75*	0.009	G03_R01_Z02_L75
	53[+]	05LoM26R4_10	BMR	G245_R01	N/A	N/A	N/A	G245_R01_X_X
	56	05LoS22_5	BMR	G03_R01	0.518	Z05_L01	0.009	G03_R01_Z05_L01
	61[+]	05LoS23R3_45	BMR	G14_R14	N/A	N/A	N/A	G14_R14_X_X
	76	05LoS1_5	BMR	G107_R02	0.007	Z29_L02	0.009	G107_R02_Z29_L02
	87	09LoS35_1	UCR	G03_R01	0.505	Z06_L11	0.167	G03_R01_Z06_L11
	131	13LoS28_1	UCR	G11_R07	0.154	Z02_L76*	0.042	G11_R07_Z02_L76
2 - March 28th 2016								
	132	05LoS3_4	BMR	G106_R04	0.014	Z02_L07	0.027	G106_R04_Z02_L07
	134	05LoS14_6	BMR	G05_R02	0.160	Z01_L07	0.009	G05_R02_Z01_L07
	135	05LoS1_7	BMR	G02_R98	0.007	Z01_L03	0.018	G02_R98_Z01_L03
	137	13LoS14_2	GP	G40_R19	0.058	Z53_L18*	0.25	G40_R19_Z53_L18
	138	13LoS15_1	GP	G91_R225	0.014	Z12_L77*	0.25	G91_R225_Z12_L77
	139	13LoS16_4	GP	G229_R226	0.014	Z61_L78*	0.25	G229_R226_Z61_L78
	141	13LoS18_3	GP	G230_R227	0.014	Z62_L79*	0.25	G230_R227_Z62_L79
	143	11LoS14_3	CLA	G71_R32	0.139	Z43_L16	0.013	G71_R32_Z43_L16
	144	11LoS13_1	CLA	G03_R34	0.013	Z02_L15	0.063	G03_R34_Z02_L15
	147	13LoS58_2	UCR	G58_R34	0.011	Z10_L80*	0.042	G58_R34_Z10_L80
	149	09LoS38R4_7	UCR	G59_R21	0.011	Z13_L23*	0.042	G59_R21_Z13_L23
	150	09LoS36_1	UCR	G03_R07	0.088	Z02_L04	0.375	G03_R07_Z02_L04
	153	11LoS33_4	YUC	G62_R19	0.025	Z13_L18	0.063	G62_R19_Z13_L18
	154	11LoS33_1	YUC	G40_R38	0.13	Z15_L34	0.250	G40_R38_Z15_L34
	155	11LoS34_2	YUC	G11_R07	0.225	Z02_L04	0.250	G11_R07_Z02_L04
	156	11LoS34_4	YUC	G03_R01	0.6	Z02_L04	0.250	G03_R01_Z02_L04
	157	11LoS31_5	ANZ	G223_R213	0.022	Z49_L25	0.023	G223_R213_Z49_L25
	159	11LoS31_1	ANZ	G62_R03	0.044	Z13_L18	0.140	G62_R03_Z13_L18
	160	12LoS21_12	ANZ	G58_R222	0.022	Z25_L21	0.047	G58_R222_Z25_L21
	162	05LoS8_14	BMR	G03_R01	0.518	Z01_L04	0.064	G03_R01_Z01_L04
	163	05LoS23_11	BMR	G03_R01	0.518	Z01_L01	0.109	G03_R01_Z01_L01

Table 1.1 Continued

Inoc group	Inoc #	Isolate	Site[#]	*glnII_recA* genotype	CHR abund[‡]	*nodZ_nolL* genotype	SI abund	Full genome genotype
3 - March 29th 2016								
	171	11LoS14_2	CLA	G03_R01	0.342	Z01_L04	0.165	G03_R01_Z01_L04
	172	11LoS18_3	CLA	G03_R01	0.342	Z02_L08	0.013	G03_R01_Z02_L08
	173	11LoS20_1	SAN	G03_R01	0.225	Z02_L07	0.025	G03_R01_Z02_L07
	174	11LoS21_3	SAN	G03_R01	0.225	Z02_L04	0.228	G03_R01_Z02_L04
	175	11LoS22_5	SAN	G73_R31	0.008	Z02_L04	0.228	G73_R31_Z02_L04
	176	11LoS28_4	CLA	G03_R01	0.342	Z01_L04	0.165	G03_R01_Z01_L04
	177	11LoS28_6	CLA	G03_R01	0.342	Z47_L04	0.013	G03_R01_Z47_L04
	178	11LoS28_7	CLA	G03_R01	0.342	Z01_L04	0.165	G03_R01_Z01_L04
	179	11LoS31_2	ANZ	G90_R27	0.044	Z15_L32	0.047	G90_R27_Z15_L32
	180	11LoS32_1	ANZ	G40_R19	0.044	Z13_L64	0.023	G40_R19_Z13_L64
	181	11LoS33_2	YUC	G40_R38	0.13	Z50_L18	0.063	G40_R38_Z50_L18
	182	11LoS33_5	YUC	G40_R38	0.13	Z13_X	0.063	G40_R38_Z13_X
	183	11LoS34_6	YUC	G03_R01	0.6	Z02_L07	0.313	G03_R01_Z02_L07
	184	11LoS34_10	YUC	G03_R01	0.6	Z02_L07	0.313	G03_R01_Z02_L07
	185	11LoS6_1	SAN	G70_R31	0.023	Z02_L04	0.038	G70_R31_Z02_L04
	187	11LoS7_1	SAN	G03_R01	0.225	Z37_L49	0.013	G03_R01_Z37_L49
	188	11LoS8_1	SAN	G03_R01	0.225	Z01_L04	0.038	G03_R01_Z01_L04
	189	12LoS6_1	SAN	G74_R219	0.008	X_L68	0.013	G74_R219_X_L68
	190	13LoS69_3	PIS	G231_R61	0.008	X_L81*	0.167	G231_R61_X_L81
	191	13LoS70_1	PIS	G74_R230	0.008	Z64_L82*	0.167	G74_R230_Z64_L82
	192	13LoS70_2	PIS	G97_R91	0.033	Z30_L39*	0.333	G97_R91_Z30_L39
	193	13LoS98_3	WHT	G11_R07	0.043	Z02_L48*	0.25	G11_R07_Z02_L48
	194	13LoS99_2	WHT	G40_R19	0.043	Z13_L18*	0.25	G40_R19_Z13_L18
	195	13LoS100_1	WHT	G243_R35	0.021	Z65_L83*	0.25	G243_R35_Z65_L83
	196	13LoS102_3	WHT	G235_R07	0.021	Z02_L69*	0.25	G235_R07_Z02_L69
	197	13LoS104_4	PIS	G102_R04	0.066	Z30_L84*	0.167	G102_R04_Z30_L84
	198	13LoS107_1	PIS	G106_R65	0.008	Z30_L39*	0.333	G106_R65_Z30_L39
	199[+]	11LoS20_4	SAN	G19_R57	N/A	N/A	N/A	G19_R57_X_X
	200	13LoS78_1	PIS	G232_R232	0.008	X_L85*	0.167	G232_R232_X_L85

Table 1.1 Continued

Inoc group	Inoc #	Isolate	Site[#]	*glnII_recA* genotype	CHR abund[‡]	*nodZ_nolL* genotype	SI abund	Full genome genotype
4 - April 3rd 2016								
	186	11LoS6_2	SAN	G210_R201	0.008	Z36_L47	0.013	G210_R201_Z36_L47
	CW1	Bulk soil	UCR	G03_R01*	0.505	Z02_L04*	0.375	G03_R01_Z02_L04
	CW3	Bulk soil	UCR	G11_R07*	0.154	Z67_L86*	0.042	G11_R07_Z67_L86
	CW4	Bulk soil	UCR	G03_R07*	0.088	Z02_L09*	0.042	G03_R07_Z02_L09
	CW5	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW7	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW8	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW9	Bulk soil	CLA	G244_R01*	0.013	Z02_L04*	0.165	G244_R01_Z02_L04
	CW10	Bulk soil	CLA	G11_R07*	0.051	Z02_L04*	0.165	G11_R07_Z02_L04
	CW11	Bulk soil	CLA	G11_R01*	0.152	Z02_L12*	0.025	G11_R01_Z02_L12
	CW12	Bulk soil	CLA	G11_R07*	0.051	Z02_L04*	0.165	G11_R07_Z02_L04
	CW13	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW14	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW15	Bulk soil	CLA	G03_R01*	0.342	Z68_L12*	0.013	G03_R01_Z68_L12
	CW16	Bulk soil	CLA	G03_R03*	0.089	Z02_L87*	0.076	G03_R03_Z02_L87
	CW17	Bulk soil	YUC	G40_R38*	0.13	Z15_L34*	0.250	G40_R38_Z15_L34

[+] Inidicates non-nodulating isolates

[#]Collection sites included Bodega Marine Reserve (BMR), Burns Piñon Ridge Reserve near Yucca Valley (YUC), Motte Rimrock Reserve (MOT), University of California Riverside (UCR), Robert J. Bernard Biological Field Station of the Claremont Colleges (CLA), Pismo Dunes Natural preserve (PIS), Whitewater Preserve (WHT), Anza Borrego Desert State Park (ANZ), Griffith Park (GP), and San Dimas (SAN).

‡CHR local genotype proportion was calculated using the total inferred nodulating isolates within a population and therefore does not include non-nodulating

*Isolates sequenced for this study

Table 1.2 Effects of non-nodulating *Bradyrhizobium* isolates

Inoc #	Site	Shoot biomass	atm%^{15}N
40	BMR	$F_{1,19} = 3.8988$	$F_{1,8} = 1.5767$
44	BMR	**$F_{1,19} = 17.8511$*** [a]**	$F_{1,8} = 1.485$
53	BMR	$F_{1,19} = 3.7816$	$F_{1,8} = 0.0401$
61	BMR	**$F_{1,19} = 5.0107$* [a]**	$F_{1,8} = 0.9570$
199	San Dimas	$F_{1,19} = 0.3997$	**$F_{1,8} = 6.7386$* [b]**

Asterisks indicate significant differences between inoculated plants and size matched controls (one-way ANOVAs; *$P <$ 0.05, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$).

[a]Refers to cases where the mean of inoculated plants is significantly *increased* compared to uninoculated controls.
[b]Refers to cases where the mean of inoculated plants is significantly *decreased* compared to uninoculated controls.

Table 1.3 Ineffective *Bradyrhizobium* isolates

Inoc #	Site	Shoot biomass	atm%^{15}N
2	BMR	**$F_{1,19}$ = 10.4678**** [a]	$F_{1,8}$ = 0.0251
155	YUC	$F_{1,20}$ = 0.0154	**$F_{1,8}$ = 21.4683**** [b]
186	SAN	$F_{1,8}$ = 1.0101	$F_{1,7}$ = 2.3456
187	SAN	$F_{1,19}$ = 0.0605	$F_{1,7}$ = 4.2916
200	PIS	$F_{1,19}$ = 1.1294	$F_{1,7}$ = 0.4060
CW1	UCR	$F_{1,8}$ = 2.7604	**$F_{1,7}$ = 14.3196**** [b]

Asterisks indicate significant differences between inoculated plants and size matched controls (one-way ANOVAs; *P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001).

[a]Refers to cases where the mean of inoculated plants is significantly *increased* compared to uninoculated controls.

[b]Refers to cases where the mean of inoculated plants is significantly *decreased* compared to uninoculated controls.

Table 1.4 Phylogenetic signal estimated with Blomberg's *K*.

Trait	***K***	**SE**	***P***
HGR	0.03834	0.00288	0.286
atm%^{15}N	0.06307	0.00732	0.164
Mean individual nodule mass	0.19232	0.00866	0.212
Local CHR abundance	0.04286	0.00202	0.263
Local SI abundance	0.02741	0.00111	0.372

Mean ±SE of *K* and average *P*-values calculated across 20 trees to account for phylogenetic uncertainty.

Table 1.5 Variation in symbiotic traits.

Trait	**Among sites**‡
HGR	**$F_{9,698}$ = 3.0575****
atm%^{15}N	$F_{9,307}$ = 1.0685
Nodule number	**$F_{9,325}$ = 3.1087****
Mean individual nodule mass	**$F_{9,324}$ = 2.0310***

Significance is denoted with asterisks (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$). Inoculated plants that failed to form nodules and uninoculated controls were excluded from analyses.

‡Effect of collection site in GLMM analyses

CHAPTER 2

Host exploitation and the maintenance of ineffective rhizobia

Abstract

Models of mutualism rest on the assumption that cheaters destabilize mutualisms. Nonetheless, there are few unequivocal examples of cheater genotypes in nature and their ecological relevance remains controversial. Cheating is modeled as a multivariate interaction trait wherein focal genotypes provide decreased benefits to partner species while gaining fitness reward from exploitation. To uncover such exploitation, both partner's fitness must be simultaneously measured in a population context, otherwise maladaptation by mismatched partners cannot be ruled out. We investigated the legume-rhizobium mutualism by quantifying fitness and fitness-effects of diverse *Bradyrhizobium* isolates upon sympatric and allopatric lines of *Acmispon* host plants. Multiple *Bradyrhizobium* genotypes were found to be nonbeneficial to hosts, and both *in planta* and population genetic measures of rhizobial fitness uncovered evidence of rhizobial exploitation. These are the first results showing that both maladaptation and host exploitation can maintain ineffective rhizobia in natural populations.

Introduction

Mutualisms are interactions in which organisms provide fitness benefits to members of other species, and biologists since Darwin have struggled to understand the evolutionary forces that maintain these associations (Darwin 1859; Bronstein 1994). Despite the mutual fitness rewards that define these interactions, natural selection works primarily at the level of the individual (Williams 1966), so cheaters that gain from the cooperation of others – but pay little or nothing in costs – are predicted to invade and threaten the collapse of mutualisms (Sachs *et al.* 2004; Sachs & Simms 2006). Models predict specific conditions in which mutualisms are maintained, including when mutualist services are cost-free (*Byproducts*; (Eberhard 1975; Brown 1983)), when fitness rewards reliably feedback to mutualists (*Partner fidelity feedback*; (Foster & Wenseleers 2006)), and when mutualists can select against cheaters (*Partner choice-Sanctions*; (Bull & Rice 1991; West *et al.* 2002a)). Selection for exploitation is a central assumption of these models, but empirical evidence for cheater genotypes has been remarkably sparse (Jones *et al.* 2015). The lack of empirical evidence of cheaters has caused biologists to question the ecological relevance of mutualism exploitation (Friesen 2012; Frederickson 2013; Kimbrel *et al.* 2013; Sachs 2015). A major hurdle in resolving this dilemma is that detecting cheating is challenging. Firstly, cheaters must be carefully differentiated from partners that fail to cooperate for non-adaptive reasons such as when erstwhile mutualists provide no benefits to genetically mismatched partners (Sachs 2015). Secondly, selection for cheating can be context dependent and thus not detected in some scenarios. For instance, in mutualisms where scarce nutrients are exchanged selection for cheating can

vary with the environmental resource base (Hoeksema et al, 2010). Moreover, in mutualisms with partner choice or sanctions, the capacity of partners to select against cheaters could vary spatiotemporally (Steidinger & Bever 2014, 2016). Finally, empirical demonstration of cheating requires fitness measures of multiple partners and interactions in a population context to clarify that cheater genotypes provide relatively less fitness rewards than conspecific mutualists while exhibiting superior fitness (Jones et al. 2015).

The legume-rhizobium mutualism is an excellent system to study mutualism exploitation because extensive phenotypic and genotypic data are available for both partner species. In this association, rhizobial bacteria form nodules on legume roots and fix costly nitrogen for their hosts in exchange for photosynthates (Sprent 1987; Lodwig *et al.* 2003). However, in many cases host legumes gain little to no fitness benefit from rhizobial nodulation. Ineffective rhizobia – nodulating genotypes that do not significantly benefit the host via nitrogen fixation – have been uncovered both in natural and agricultural populations (Quigley *et al.* 1997; Moawad *et al.* 1998; Burdon *et al.* 1999; Denton *et al.* 2000; Chen *et al.* 2002; Collins *et al.* 2002; Sachs *et al.* 2010a) and stand as a major hurdle to the improvement of legume growth (Triplett & Sadowsky 1992; Yates *et al.* 2011). Legumes exhibit sanctions traits that can limit the effects of ineffective rhizobia by constraining the *in planta* growth of ineffective rhizobia. Specifically, nodules with nitrogen-fixing rhizobia grow (and the rhizobia within them proliferate), whereas nodules with ineffective rhizobia tend to remain small (and the rhizobia within them attain reduced fitness) (Singleton & Stockinger 1983; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010b; Oono *et al.* 2011; Regus *et al.* 2014). But several studies

have failed to find evidence for host sanctions and the mechanisms underlying these results remain unclear (Heath & Tiffin 2009; Marco *et al.* 2009; Gubry-Rangin *et al.* 2010; Marco *et al.* 2015).

The maintenance of ineffective rhizobia remains a dilemma, given the apparent efficiency of sanctions in some hosts (Singleton & Stockinger 1983; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010b; Oono *et al.* 2011; Regus *et al.* 2014). Two contrasting hypotheses frame the persistence of ineffective rhizobia, which we term the maladaptation and exploitation frameworks. The maladaptation framework predicts that ineffective rhizobia are 'defective' rather than 'defectors' and pose no threat against the stability of the legume-rhizobium mutualism (Friesen 2012)). Most generally ineffectiveness can evolve under selection-mutation balance, wherein non-fixing rhizobia are continuously generated by mutation events but are slowly purged from populations by negative selection (Van Dyken *et al.* 2011). More specifically, ineffectiveness can occur as a context dependent trait. The nitrogen fixed by rhizobial genotypes can vary among host genotypes, hence that ineffectiveness can occur as a maladapted outcome expressed when rhizobia encounter an atypical host (i.e., G×G interactions; (Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Heath 2010)). In contrast, the exploitation framework predicts that ineffective rhizobia evolve adaptively to exploit host resources while selfishly minimizing costs of nitrogen fixation (i.e., cheater genotypes; (West *et al.* 2002a; West *et al.* 2002b; Ghoul *et al.* 2014; Jones *et al.* 2015; Sachs 2015). A prominent prediction under the exploitation framework is that conditions of nitrogen rich soil can lead to downregulation or relaxed selection on sanctions, wherein exploitative rhizobia

can invade (Kiers *et al.* 2006; Kiers *et al.* 2007; Weese *et al.* 2015). Moreover, host sanctions are predicted to vary in a spatiotemporally in a manner that depends on the local frequency of beneficial rhizobial partners, thus generating potential for a local refuge in which ineffective rhizobial genotypes can be maintained (Steidinger & Bever 2014, 2016).

Here, we investigate the maintenance of ineffective rhizobia in a metapopulation of native hosts and symbionts that experience spatial structure in soil nitrogen availability. *Acmispon strigosus* (formally *Lotus strigosus*) is an annual legume native to the Southwestern USA that is nodulated by *Bradyrhizobium spp*. that range from highly beneficial to ineffective (Sachs *et al.* 2010a). We analyzed symbiotic effectiveness and fitness proxies for thirty genetically diverse *Bradyrhizobium* isolates upon a variety of genotypically diverged hosts (Hollowell *et al.* 2016a; Hollowell *et al.* 2016b). *Bradyrhizobium* were collected from six *A. strigosus* populations across California that exhibit population structure in their *Bradyrhizobium* communities (Hollowell *et al.* 2016a; Hollowell *et al.* 2016b) and experience ~10× variation in soil nitrogen concentrations (Fenn *et al.* 2010; Regus *et al.* 2014). We performed clonal inoculations of each *Bradyrhizobium* genotype onto five *Acmispon* host treatments, including two sympatric *A. strigosus* plant lines, two universal *A. strigosus* lines, and mixed seed sets of *A. heermannii*, a sympatric sister taxon (Allan & Porter 2000). Three main questions were investigated: (i) Do wild *A. strigosus* hosts frequently encounter ineffective rhizobia? (ii) Does rhizobial effectiveness vary consistently with host genotype or soil nitrogen and (iii) Do ineffective rhizobia exhibit evidence of superior fitness relative to

sympatric effective rhizobia, either *in planta* or in the context of local genotype abundance? This work explicitly tests hypotheses underlying the maladaptation and exploitation frameworks, and thus our data provides insight into the mechanisms that maintain ineffective rhizobia in natural populations.

Materials and Methods

Field sites and *Bradyrhizobium* isolation

Bradyrhizobium spp. were previously isolated from the nodules and the soil-root interface of *A. strigosus* host plants at natural field sites across California (Sachs *et al.* 2009; Hollowell *et al.* 2016a). Here, isolates from six focal sites were selected for further analyses wherein three locales exhibit low levels of soil nitrogen (~2-7 ppm mineral nitrogen; Bodega Marine Reserve, BMR; Burns Piñon Ridge Reserve near Yucca Valley, YUC; Anza Borrego Desert State Park, ANZ) and three have higher nitrogen soils impacted by anthropogenic nitrogen deposition (~7-20 ppm mineral nitrogen; Griffith Park, GIR; Bernard Biological Field Station of the Claremont Colleges, CLA; University of California Riverside, UCR) (Regus *et al.* 2014). At all sites whole plants were excavated from the soils and brought back to the lab. Roots were washed with tap water to remove soil. Nodules were dissected from the roots using sterile tools, surface sterilized in bleach (5% sodium hypochlorite), and rinsed in sterilized water. Nodules were individually crushed with a sterile glass rod, contents plated onto modified arabinose gluconate (MAG) agar plates, and a single rhizobial colony archived (Sachs *et al.* 2009). For a subset of plants, *Bradyrhizobium spp.* were cultured from the soil root interface. For these plants the root systems were dissected into 1 cm sections, placed into tubes with sterile ddH_20 wash solution, vortexed, and the solution was serially diluted and plated on a glucose-based rhizobium defined media (Sachs *et al.* 2009). *Bradyrhizobium* were selected from the resultant colonies based on growth on growth

rate and color selective media and were later genotyped (Sachs *et al.* 2009; Hollowell *et al.* 2016a).

Bradyrhizobium genotyping

Published genotyping data from the sympatric *Bradyrhizobium* populations were used to infer relative fitness of different *Bradyrhizobium* genotypes. A collection of 1292 *Bradyrhizobium* nodule and root surface isolates from fourteen natural field sites were previously sequenced at two chromosomal loci (*glnII* and *recA*) and assigned to chromosomal genotypes based on both loci (i.e. CHR genotype; (Hollowell *et al.* 2016a)). Sequences from each locus were aligned separately using Clustal Omega (Maddison & Maddison 2005; Sievers *et al.* 2011) and isolates with identical genotypes were identified using the "find redundant" command in MacClade (Maddison & Maddison 2005). A subset of these isolates, collected from nine of the fourteen sites (358 isolates), were additionally sequenced at the 'symbiosis island' loci *nodZ* and *nolL* and assigned to symbiosis island genotypes based on these loci (i.e., SI genotype; (Hollowell *et al.* 2016b)). The symbiosis island is a large genomic island that is integrated into the *Bradyrhizobium* genome and encodes for nodulation and nitrogen fixation functions (Kaneko *et al.* 2002). Local genotype abundance was calculated for each genome region (CHR, SI) for each tested isolate, which is the proportion of the focal genotype among the total number of nodulating isolates in a sampled population.

Focal Bradyrhizobium isolates

Thirty genetically diverged *Bradyrhizobium* isolates were chosen for this study, with 4-6 isolates selected from each of the six field sites (**Table 2.1**). All isolates were previously assigned CHR genotypes (Hollowell *et al.* 2016a) and the majority of isolates were previously assigned SI genotypes (Hollowell *et al.* 2016b). Isolates were selected in an attempt to sample a broad range in CHR and SI genotype abundance (where data was available). Isolates without previously assigned SI genotypes (#'s 137, 138, 139, 141, 147, 149, and 152) were initially chosen based on collection site and CHR genotype abundance. For isolates with missing SI data, genomic DNA was PCR amplified and sequenced at the Institute for Integrative Genome Biology of UC Riverside following published protocols (Stępkowski *et al.* 2005; Hollowell *et al.* 2016b). Additional sequence data was added to the SI dataset and SI genotypes were determined as described previously.

Acmispon hosts

Fourteen lines of *A. strigosus* were used for inoculation experiments, including two lines from each field site to be inoculated with sympatric *Bradyrhizobium* isolates (i.e., sympatric hosts). Two additional lines served as universal hosts for all thirty *Bradyrhizobium* isolates (**Table 2.2**). One universal line originated from a site with high concentrations of soil nitrogen (CLA; 'UnH' line Cla12.04) and the other was from a low nitrogen site (ANZ; 'UnL' line Anz13.04). *Acmispon strigosus* plant lines were descended from wild collected seeds and generated following published methods (Wendlandt et al. 2017). Plants for seeds used in this study were grown for 5 months

(November 2013 – April 2014) and ripe fruits were picked as they matured. Additionally, we used mixed seed sets of *A. heermannii* host plants from a single local source in Hemet, CA (S&S Seeds, Carpinteria, CA). *Acmispon heermannii* is a close relative of *A. strigosus* (Porter *et al.* 2000) that is broadly sympatric throughout California (**www.calflora.org)**.

Inoculation experiments

Bradyrhizobium isolates were grown from original frozen stocks and streaked onto agar plates containing modified arabinose gluconate medium (MAG) (Sachs *et al.* 2009), and a single colony was spread onto five replicate MAG plates and incubated until lawns formed (29°C, ~8 days). Bacterial cells were washed from plates and resuspended in liquid MAG to estimate concentrations via optical density (Sachs *et al.* 2010a). Resuspended cells were then centrifuged (4000 rpm, 20 minutes) to remove media and resuspended again in sterile ddH_2O at 10^8 cells ml^{-1}. Inoculated plants received 5×10^8 rhizobial cells in 5ml of sterile ddH_2O and uninoculated controls received 5ml of sterile ddH_2O.

Seeds were surface sterilized, nick scarified, and germinated in sterile nitrogen-free Jensen's solution (Somasegaran & Hoben 2012). Seedlings were planted into sterilized conetainers (Steuwe and Sons, Tangent, OR, USA) filled with sterile quartzite sand and incubated in a growth chamber for two weeks. Seedlings were then moved to a greenhouse under ~50% shade for hardening (4 days, 1 × daily misting). Plants were

fertilized weekly with 1ml of sterile nitrogen-free Jensen's solution increasing by 2ml per week until reaching 5ml per plant, which was used for the duration of the experiment.

For each host line and species, axenic seedlings were arranged by size and groups of sized matched seedlings were randomly assigned to inoculation treatments and greenhouse locations. Each *Bradyrhizobium* treatment was paired with control inoculations of sterile ddH_2O for each host population source. Each treatment was replicated on 5 plants separated into individual blocks (36 bacterial treatments × 5 host lines per treatment × 5 replicates per treatment, except for lines UnL, Anz10.01, and Gri01.13 which had 4 replicates = 852 plants). Plants were inoculated on March 13th 2015 and harvested eight weeks after inoculation. During harvest, plants were removed from the pots and soil was separated from the roots by washing with tap water (May 13th – May 26th 2015). Plants were individually wrapped and stored on trays at 4°C until dissection (May 13th – June 11th 2015). Nodules were dissected, counted, and photographed. Roots, shoots, and nodules were separated and oven dried (60°C, >4 days) prior to weighing.

Data Analysis

Nodulation capacity of each *Bradyrhizobium* isolate was assessed by the presence or absence of nodules on tested hosts. Symbiotic effectiveness was estimated as the inoculated host's growth response to *Bradyrhizobium* inoculation relative to the growth of size-matched uninoculated controls (HGR = [(Shoot mass of Inoculated Plant – Shoot mass of Control Plant)/Shoot mass of Control Plant] × 100; (Sachs *et al.* 2010a)).

Previous work showed HGR to be good proxy for symbiotically fixed nitrogen (Regus *et al.* 2014). Nodulating isolates were classified as ineffective if they did not cause a significant aboveground growth response relative to uninoculated controls (measured using one-way ANOVA followed by Tukey's HSD test). We used mean nodule mass to estimate rhizobial fitness *in planta*, which is positively correlated with rhizobial population sizes in *A. strigosus* nodules (Sachs *et al.* 2010a) and other species such as *Medicago truncatula* (Heath & Tiffin 2007, 2009), *Glycine max* (Kiers *et al.* 2003), and *Lupinus arboreus* (Simms *et al.* 2006). Host growth response and mean nodule mass of ineffective isolates were compared to the population means using a one sample *t*-test. Local genotypic abundance of ineffective isolates was also compared to the abundance of the most effective isolate from the same population.

Generalized linear mixed models (GLMMs) were used to analyze variation among host populations, with population source coded as a fixed effect and isolate coded as a random effect. Variation in symbiotic effectiveness (i.e., HGR) and rhizobial fitness among isolates were analyzed using ANOVAs. GLMMs were used to test for interaction effects between host and rhizobial genotype and effects of local soil nitrogen on symbiotic effectiveness. In the host genotype models, host line, isolate, and their interaction were treated as fixed effects. In the soil nitrogen models, field site of origin was treated as a fixed effect and isolate as a random effect. Effects of mineral and total nitrogen in soils were also examined using a GLMM with mineral N and total N as fixed effects and isolate as a random effect. For both host genotype and soil nitrogen models, a

more stringent analysis was also performed including only universal *A. strigosus* host lines allowing for a full factorial design.

GLMMs were run separately for each host type (i.e., sympatric, UnH, UnL, and *A. heermannii*) to test for effects of symbiotic effectiveness on nodule mass. Mean nodule mass was treated as a fixed effect and isolate as a random effect. On sympatric hosts, separate GLMMs for each genomic region were used to test the effects of symbiotic effectiveness on local genotypic abundance. For each genomic region, local genotypic proportion was treated as fixed effect and isolate as a random effect. All models were analyzed using the Fit Model Platform in JMP v. 10.0 (Inc. 2012) and response variables were log transformed to improve normality when necessary. ANOVAs or GLMMs with significant *F* ratio statistics were followed by Tukey's HSD test to test for differences among treatments.

Results

Nodulation capacity

Eight-hundred and forty-five plants (of 852) survived the duration of the experiment. Twenty-two of the thirty tested *Bradyrhizobium* isolates formed nodules on all surviving inoculated plants and twenty-six of the isolates formed nodules on most inoculated plants. Isolate #'s 134, 135, and 158 each failed to nodulate a single plant replicate. Isolate #149 exhibited inconsistent nodulation on both sympatric lines and on 'UnL' hosts. Four isolates failed to nodulate any hosts (#'s 133, 140, 148, and 161) and failed to amplify the SI loci. Isolate #'s 133 and 148 were originally isolated from the *A. strigosus* root surface. Isolate #'s 140 and 161 were originally isolated from nodules, but failed to nodulate any hosts, suggesting that they might have coinfected the original host with a nodulating isolate (i.e., (Rangin *et al.* 2008; Gano-Cohen *et al.* 2016). Inoculated plants that did not form nodules were removed from remaining analyses. None of the uninoculated control plants formed nodules (**Table S2.1**).

Variation in symbiotic traits

Symbiotic effectiveness did not vary significantly between sympatric host lines within populations, so these data were combined for subsequent analyses (**Table S2.2**). Symbiotic effectiveness varied significantly among isolates and host populations. There were no significant differences in symbiotic effectiveness among isolates within most populations, with the exception of the YUC population (ANOVAs with uninoculated controls removed) (**Table 2.3**; **Figure 2.1a**). Three novel ineffective isolates were

uncovered, including isolate #149 from UCR and #'s 155 and 156 from YUC (**Figure 2.1a**). Mean nodule mass varied significantly among isolates on sympatric hosts and among host populations (**Table 2.3**; **Figure 2.1b**).

Symbiotic effectiveness exhibited significant variation on the universal line 'UnH' and on *A. heermannii* hosts, but not on universal line 'UnL' (**Table 2.3; Figures S2.1-2.3a**). Mean nodule mass among isolates varied within all universal hosts (**Table 2.3; Figures S2.1-2.3b**).

Testing hypotheses for the maintenance of ineffective rhizobia

We did not find evidence that host specificity (i.e., G×G interactions) maintains ineffective *Bradyrhizobium* in populations. Ineffective isolates, initially defined by tests on sympatric hosts, were consistently categorized as ineffective irrespective of host genotype (**Figures S2.1-2.3a**). Moreover, analysis of all *Acmispon* hosts showed that symbiotic effectiveness did not exhibit a significant G×G interaction despite significant effects of host genotype and isolate (**Table 2.4**). Analyses focused only on universal hosts demonstrated similar results (see **Table S2.3**).

All ineffective isolates significantly reduced host growth below the population mean on sympatric hosts (**Figure 2.1a**) and formed nodules with mass equal to (#155), or greater than (#'s 149, 156) the most effective isolate from their respective populations (**Figure 2.1b**) and this pattern was consistent on universal hosts (**Figures S2.1-2.3b**). Only isolate #156 exhibited significantly higher mean nodule mass on sympatric hosts compared to the population mean (**Figure 2.1b**). Symbiotic effectiveness did not exhibit

a significant effect of mean nodule mass, either on sympatric or universal *Acmispon* hosts (**Table 2.4**).

Local genotypic abundance of ineffective isolates was equal to (#149) or greater (#'s 155, 156) than the most effective isolate from the same population and this was true for both genome regions (CHR and SI; **Figure 2.1c, d; Table 2.1**). Only isolate #156 had a genotype abundance above the population mean for both genome regions. Isolate #152 previously did not reliably amplify both SI loci used in this study (Hollowell *et al.* 2016a) and therefore was not included in analyses of the SI loci in the present study.

Mean symbiotic effectiveness was negatively correlated with local genotypic abundance for both the CHR and SI loci, hence that the more locally abundant genotypes provided less benefit to sympatric hosts on average (CHR: $F_{1,247} = 4.5497$, $P = 0.0432$; SI: $F_{1,237} = 4.6492$, $P = 0.0417$) (**Figure S2.4**). Symbiotic effectiveness was not correlated with local abundance for the full CHR+SI genotypes ($F_{1,247} = 1.6700$, $P = 0.2086$). However, this dataset contained a large proportion of locally unique genotypes (see **Figure S2.4**).

We did not find evidence that variation in soil nitrogen maintains ineffective *Bradyrhizobium* in populations. We found a significant effect of rhizobial field site of origin on symbiotic effectiveness ($F_{5,468} = 3.1581$, $P = 0.0290$; all *A. strigosus* hosts analyzed). However, these site effects were not structured by nitrogen deposition rates (i.e., 'high' versus 'low' sites; Fenn 2010) (Contrast analysis; $F_{1,472} = 1.9740$, $P = 0.1753$), or by soil measures of total N ($F_{1,472} = 2.0614$, $P = 0.1646$) or mineral N ($F_{1,472}$

= 2.1854, $P = 0.1526$) (Regus 2014). We found no effects of rhizobial field site of origin when only universal hosts were analyzed (**Table S2.4**).

Discussion

Models of mutualism maintenance rest on the assumption that selection can favor cheating, but relatively little is known about how often cheater mutants arise or the conditions under which cheaters can persist (Ghoul *et al.* 2014; Jones *et al.* 2015; Sachs 2015). Understanding the patterns and drivers of ineffective rhizobia will not only provide critical insights into the maintenance of mutualist variation in this system, but will also enhance agricultural practices that apply rhizobial soil amendments (Denison 2000; Denison & Kiers 2004; Sachs & Simms 2008).

Empirical evidence of ineffective rhizobia has been uncovered in multiple host species and environmental contexts, yet measures of ineffectiveness vary, making initial comparisons among datasets challenging (Quigley *et al.* 1997; Moawad *et al.* 1998; Burdon *et al.* 1999; Denton *et al.* 2000; Chen *et al.* 2002; Collins *et al.* 2002; Sachs *et al.* 2010a). Ineffective rhizobia have been defined relative to beneficial rhizobia (as in Burdon *et al.* 1999), or to uninoculated controls (as in Sachs *et al.* 2010a), or in relation to both (i.e., 'relative effectiveness' as in (Bromfield *et al.* 2010). Fortunately, even if these definitions are not explicitly used, most studies utilize either uninoculated controls (as in Sachs *et al.* 2010a) or defined beneficial reference strains (as in Burdon *et al.* 1999). Appropriately applying either of these definitions to published datasets (Sachs *et al.* 2010a for studies including uninoculated controls and Burdon *et al.* 1999 for studying lacking uninoculated controls; as performed in **Table S2.5**) allows for general comparisons among studies as well as broader surveys of rhizobial ineffectiveness.

Ineffective rhizobial seem to be generally more common in agricultural soils compared to unmanaged soils. In agricultural studies, ineffective rhizobia range broadly in their recovered frequencies, composing 2-95% of tested isolates and have been uncovered in 11-88% of surveyed host populations (Gibson *et al.* 1975; Quigley *et al.* 1997; Moawad *et al.* 1998; Denton *et al.* 2000; Chen *et al.* 2002; Collins *et al.* 2002; Fening & Danso 2002; Rangin *et al.* 2008; Bromfield *et al.* 2010) (**Table S2.5**). However, in studies that utilized multiple legume host cultivars (Collins *et al.* 2002) or host species (Denton *et al.* 2000; Rangin *et al.* 2008), ineffective rhizobia were not consistently ineffective on all hosts (but see (Bromfield *et al.* 2010; Simonsen & Stinchcombe 2014)) suggesting that failure to fix nitrogen is often driven by nodulation of an atypical host (i.e., G×G interactions). Fewer studies have investigated ineffective rhizobia from unmanaged soils, but ineffective rhizobia have been shown to compose only up to 27% of assayed rhizobial isolates (Burdon *et al.* 1999; Sachs *et al.* 2010a; Ehinger *et al.* 2014). Despite the suggested reduced incidence of ineffective rhizobia in natural soils, ineffective rhizobia have been recurrently found among disparate geographic regions (Gaur & Lowther 1980) or host species collected from multiple natural populations (Burdon *et al.* 1999) (**Table S2.5**). In contrast to agricultural studies, ineffective rhizobia isolated from conspecific unmanaged hosts tend to be constitutively ineffective when inoculated onto different host genotypes (Burdon *et al.* 1999; Sachs *et al.* 2010a; Regus *et al.* 2015) or onto another host species (Ehinger *et al.* 2014). In the present study, the absolute number of *Bradyrhizobium* isolates tested is relatively small. Nonetheless we found that ~10% of the nodulating isolates were ineffective and that

ineffectiveness was a constitutive trait expressed in all host genotypes tested. These results are consistent with other studies of unmanaged soils and suggests ineffective rhizobia may be frequent enough to contribute to the maintenance of legume sanctions in natural populations (see (Frederickson 2013)).

Although we found consistent patterns among two proxies of rhizobial fitness (mean nodule mass and genotypic abundance), both of these metrics have limitations in regards to understanding the effects on rhizobial fitness. Mean nodule mass is often positively correlated with rhizobial fitness in *A. strigosus* nodules (Sachs *et al.* 2010a; Regus *et al.* 2015), but may not result in a direct increase in rhizobial fitness within the population under natural conditions (i.e., may not represent a direct fitness benefit) (Friesen 2012). Moreover, single inoculation studies can mask the expression of host control traits, such as preferentially rewarding beneficial symbionts and punishing uncooperative genotypes, as compared to more natural environments in which hosts encounter multiple rhizobial genotypes that vary in effectiveness. We also used local genotype abundance as fitness proxy, as quantifying the relative frequency of a genotype in a population can uncover recent clonal expansion events that indicate superior fitness (Hollowell *et al.* 2016a; Hollowell *et al.* 2016b). While our estimates of genotype abundance encompass both an extensive geographic range and multiple populations, the sampling is still relatively sparse and a more intensive population genomic approach will substantially contribute to our understanding of population dynamics of ineffective genotypes in natural environments. Lastly, it is important to note that we often tested only one isolate per genotype (at the 4 loci included in this study) and it is possible that

different isolates within a genotype express different symbiotic phenotypes (Bromfield *et al.* 1987; Fuhrmann 1990; Hartmann & Amarger 1991; Hollowell *et al.* 2016a). While the genotypic abundance data are useful for analyzing fitness variation across the dataset, additional work is needed to quantify the fitness of any single genotype.

Multiple legume species exhibit sanctions traits that limit the impact of ineffective rhizobia (Singleton & Stockinger 1983; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010b; Oono *et al.* 2011; Regus *et al.* 2014) and population models predict the extirpation of ineffective genotypes under the expression of host sanctions (Denison 2000; West *et al.* 2002a; West *et al.* 2002b). Yet, there are several reasons for why sanctions might be unsuccessful in eliminating ineffective rhizobia in populations. Firstly, symbiotic effectiveness can be host genotype dependent. Although our data does not support the hypothesis that host genotype interactions (G×G) are responsible for the maintenance of *ineffective* rhizobia in natural soils (**Figure 2.1a; Figures S2.1-2.3a**), several studies have found evidence that G×G interactions influence overall rhizobial quality and suggest theses interactions are common in nature (Bever 1999; Burdon *et al.* 1999; Heath & Tiffin 2007; Laguerre *et al.* 2007; Heath & Tiffin 2009; Heath 2010; Ehinger *et al.* 2014). In contrast, our data suggests that ineffective *Bradyrhizobium* may be constitutively ineffective and suggests context dependency and maladaptation are not sole drivers for the maintenance of ineffective rhizobia.

Alternatively, sanctions may not eliminate ineffective rhizobia if host genotypes can vary in sanctioning ability, thereby generating spatially or temporally variable selection on symbiont populations (Foster & Kokko 2006; Steidinger & Bever 2014,

2016). Models of host variation in sanctions often assume hosts incur a cost to bearing or expressing sanctioning traits and predict that sanctions are downregulated or lost when the benefits of symbiosis do not outweigh these costs. One example is the prediction that sanctions are degraded in nitrogen rich soils (Kiers *et al.* 2007), wherein hosts might gain marginal or no benefits from nodulation (Regus *et al.* 2014; Regus *et al.* 2015). Consistent with these models, rhizobia isolated from soils under an experimental long-term (22 year) nitrogen fertilizer regime exhibited reduced symbiotic effectiveness compared to control plots with unfertilized soils (Weese *et al.* 2015). However, in this study they observed a reduction in the frequency of legume hosts in the fertilized plots, which could also relax selection for isolate effectiveness independent of soil nitrogen levels (Sachs & Simms 2008). In contrast, our study used diverged host genotypes from natural populations and did not find that symbiotic effectiveness varied with local soil nitrogen. Spatiotemporal variation in host sanctions may also be driven by the local frequency of ineffective rhizobia. Assuming a significant cost of sanctioning, non-sanctioning hosts may have higher fitness when the incidence of ineffective rhizobia is low (Steidinger & Bever 2014, 2016). This might facilitate the invasion of ineffective rhizobia within a host population, but would eventually lead to selection for sanctioning hosts once ineffective genotypes became common. The resulting dynamic equilibrium between sanctioning and non-sanctioning host populations, and effective and ineffective symbionts can simultaneously limit the evolutionary spread of ineffective symbionts, but also maintain them within some populations. Supporting this model, ineffective genotypes only dominated in one of the examined host populations in this study (YUC;

Figure 2.1). Not only does this suggest YUC might be a non-sanctioning host population and thus act as a reservoir maintaining ineffective rhizobia, but it also implies that sanctions traits among *A. strigosus* populations may be spatially variable.

Finally, some ineffective rhizobia might be able to overcome sanctioning mechanisms. Using nodule mass as a fitness proxy, two single inoculation studies have demonstrated evidence of ineffective rhizobia subverting legume sanctions, wherein ineffective rhizobia had higher nodule mass compared to effective isolates (soybean; (Abd-Alla 1992) and pea; (Lodwig *et al.* 2003)). Moreover, recent work in the wild *Medicago-Ensifer* mutualism uncovered a selection gradient that favors cheating in rhizobia, suggesting selection should favor rhizobia that fix less nitrogen, but exploitative genotypes were not directly identified (Porter & Simms 2014). Despite the apparent efficiency of *A. strigosus* host sanctions (Sachs *et al.* 2010b; Regus *et al.* 2014), we found evidence consistent with the host exploitation framework for the maintenance of ineffective rhizobia in natural populations. Specifically, we clonally inoculated a diverse suite of wild *Bradyrhizobium* isolates and uncovered ineffective isolates that do not provide significant benefit to hosts and have fitness equal to or greater than effective isolates. Both of our estimates of rhizobial fitness suggest ineffective isolates recovered here can potentially overcome host sanctioning mechanisms in single inoculation experiments, and may have evolved adaptively to exploit host resources. However, single inoculation studies are not directly applicable to nature, wherein hosts legumes are often infected with multiple rhizobial genotypes and ineffective isolates are sanctioned (Sachs *et al.* 2010b). Future coinoculation experiments are needed to confirm that ineffective

isolates uncovered here are successful exploiters in more natural settings. It is also unclear if uncovered ineffective isolates evolved to be ineffective and then spread throughout symbiont populations or if ineffectiveness was independently and recurrently evolved.

In summary, we found that ineffective rhizobial genotypes are easily recovered in natural *A. strigosus* populations. There was no evidence that host specificity acts as a key driver of ineffectiveness, suggesting that these genotypes are not maladapted under the tested conditions. We also did not find an effect of soil nitrogen levels on the presence or effectiveness of rhizobial symbionts. Instead, our rhizobial fitness data supports the exploitation framework and suggests that ineffective isolates may have evolved adaptively to exploit host resources. Importantly, we identified spatial variation in rhizobial cheating, as much of our evidence came from a single host population. Future efforts that examine the spatiotemporal patterns of rhizobial cheating and host sanctions will reveal whether these patterns are driven by ongoing coevolutionary conflict.

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Figure 2.1 Variation in symbiotic traits on sympatric hosts. Data from both sympatric lines within a population were combined. Significant differences among isolates within a population are indicated with lowercase letters (one-way ANOVA followed by Tukey's HSD test). Significant differences among host populations are indicated with capital letters (GLMM). The cross symbol (†) denotes ineffective isolates. For populations where ineffective rhizobia were identified, red dashed lines represent the population mean and asterisks indicate significant differences from the population mean (one sample *t*-test, $P < 0.05$). (a) Relative growth. (b) Individual nodule mass. (c) Chromosomal and (d) Symbiosis island genotype abundance represented as a proportion of nodulating isolates within a population. Error bars represent 1 standard error.

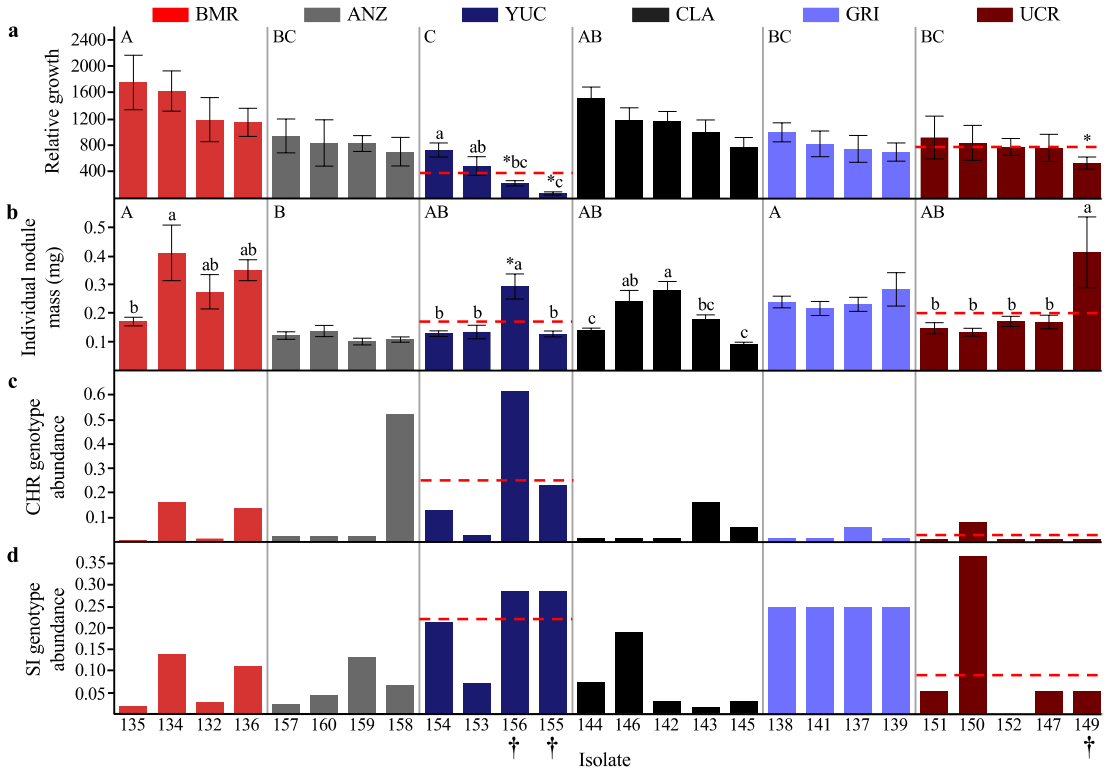

Table 2.1 Summary of isolate information.

Population	Inoculation #	Strain Name	CHR genotype	CHR abundance	SI genotype	SI abundance	Genome haplotype	Genome abundance
Bodega Marine Reserve (BMR)								
	132	05LoS3_4	G106_R04	0.0146	Z02_L07	0.0278	G106_R04_Z02_L07	1
	133*	05LoS14R9_26	G84_R100	N/A	N/A	N/A	G84_R100_X_X	N/A
	134†	05LoS14_6	G05_R02	0.1606	Z01_L07	0.1389	G05_R02_Z01_L07	14
	135†	05LoS1_7	G02_R98	0.0073	Z01_L03	0.0185	G02_R98_Z01_L03	1
	136	05LoS1_4	G01_R01	0.1387	Z01_L01	0.1111	G01_R01_Z01_L01	9
Griffith Park (GRI)								
	137	13LoS14_2	G40_R19	0.0588	Z53_L18‡	0.25	G40_R19_Z53_L18	1
	138	13LoS15_1	G91_R225	0.0147	Z12_L77‡	0.25	G91_R225_Z12_L77	1
	139	13LoS16_4	G229_R226	0.0147	Z61_L78‡	0.25	G229_R226_Z61_L78	1
	140*	13LoS10_1	G82_R55	N/A	N/A	N/A	G82_R55_X_X	N/A
	141	13LoS18_3	G230_R227	0.0147	Z62_L79‡	0.25	G230_R227_Z62_L79	1
Robert J. Bernard Biological Field Station (CLA)								
	142	11LoS29_1	G71_R212	0.0147	Z20_L16	0.0294	G71_R212_Z20_L16	1
	143	11LoS14_3	G71_R32	0.1618	Z43_L16	0.0147	G71_R32_Z43_L16	1
	144	11LoS13_1	G03_R34	0.0147	Z02_L15	0.0735	G03_R34_Z02_L15	1
	145	11LoS15_1	G11_R05	0.0588	Z19_L08	0.0294	G11_R05_Z19_L08	2
	146	11LoS17_12	G72_R01	0.0147	Z02_L04	0.1912	G72_R01_Z02_L04	1
University of California Riverside (UCR)								
	147	13LoS58_2	G58_R34	0.0114	Z10_L80	0.0526	G58_R34_Z10_L80	1
	148*	09LoS35R1_21	G22_R80	N/A	N/A	N/A	G22_R80_X_X	N/A
	149†	09LoS38R4_7	G59_R21	0.0114	Z13_L23	0.0526	G59_R21_Z13_L23	1
	150	09LoS36_1	G03_R07	0.0795	Z02_L04	0.3684	G03_R07_Z02_L04	1
	151	09LoS36_3	G03_R159	0.0114	Z07_L04	0.0526	G03_R159_Z07_L04	1
	152	09LoS38R12_17	G04_R07	0.0114	N/A	N/A	G04_R07_X_X	
Burns Piñon Ridge Reserve (YUC)								
	153	11LoS33_4	G62_R19	0.0256	Z13_L18	0.0714	G62_R19_Z13_L18	1
	154	11LoS33_1	G40_R38	0.1282	Z15_L34	0.2143	G40_R38_Z15_L34	3
	155	11LoS34_2	G11_R07	0.2308	Z02_L04	0.2857	G11_R07_Z02_L04	2
	156	11LoS34_4	G03_R01	0.6154	Z02_L04	0.2857	G03_R01_Z02_L04	2
Anza Borrego Desert State Park (ANZ)								
	157	11LoS31_5	G223_R213	0.0227	Z49_L25	0.0222	G223_R213_Z49_l25	1
	158	12LoS20_1	G58_R27	0.5227	Z15_L37	0.0667	G58_R27_Z15_L37	3
	159	11LoS31_1	G62_R03	0.0227	Z13_L18	0.1333	G62_R03_Z13_L18	1
	160	12LoS21_12	G58_R222	0.0227	Z25_L21	0.0444	G58_R222_Z25_L21	1
	161	12LoS20_4	G60_R220	N/A	N/A	N/A	G60_R220_X_X	N/A

*Non-nodulating isolates

†Isolates with inconsistent nodulation

‡SI loci sequenced in this study

Table 2.2 *Acmispon* hosts and inoculation treatments

Population	Inoculation Strains	Host line name	Host line number
Bodega Marine Reserve (BMR)			
	132-136		
		AcS074.BMR.u01.g2.r01_03	BMR01.03
		AcS004.BMR.u01.g2.r01_03	BMR07.03
Griffith Park (GRI)			
	137-141		
		AcS075.Gri.u01.g1.r01	Gri01.01
		AcS075.Gri.u01.g1.r13	Gri01.13
Robert J. Bernard Biological Field Station (CLA)			
	142-146		
		AcS047.Cla.m01.g2.r07_01	Cla10.01
		AcS049.Cla.m01.g1.r04	Cla01.04
University of California Riverside (UCR)			
	147-152		
		AcS027.UCR.u01.g1.r10	UCR02.07
		AcS131.UCR.u01.g1.r05	UCR09.05
Burns Piñon Ridge Reserve (YUC)			
	153-156		
		AcS052.Yuc.m01.g2.r01_07	Yuc02.07
		AcS052.Yuc.m01.g2.r01_01	Yuc02.01
Anza Borrego Desert State Park (ANZ)			
	157-161		
		AcS040.Anz.m01.g2.r06_01	Anz11.01
		AcS039.Anz.m01.g2.r03_01	Anz10.01
Universal			
	ALL		
		AcS047.Cla.m01.g2.r09_04	UnH: Cla12.04
		AcS038.Anz.m01.g1.r11	UnL: Anz13.04
		A. heermannii	mixed seed set

Table 2.3 Variation in symbiotic traits

	Symbiotic effectiveness	Mean nodule mass
Sympatric hosts		
Among isolates†	$F_{25,223}$ = 3.1439****	$F_{25,220}$ = 5.7294****
Among host populations‡	$F_{5,243}$ = 8.2364***	$F_{5,240}$ = 3.7807*
Universal hosts†		
UnH	$F_{25,103}$ = 1.7336*	$F_{25,123}$ = 2.5503***
UnL	$F_{25,72}$ = 1.5319	$F_{25,72}$ = 42.3563****
A. heermannii	$F_{25,104}$ = 1.6149*	$F_{25,123}$ = 2.8369****

Significance is denoted with asterisks (*P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001). Inoculated plants that failed to form nodules were excluded from analyses

†Analyzed using ANOVAs

‡Effect of population in GLMM analysis

Table 2.4 Maintenance of ineffective rhizobia. Effects of isolate, host genotype, mean nodule mass, and abundance on symbiotic effectiveness.

Maladaptation hypothesis†				
	Symbiotic effectiveness			
Isolate	$F_{25,577}$ = 7.4874****			
Host genoptype	$F_{4,598}$ = 18.7829****			
Isolate × Host genotype	$F_{100,502}$ = 0.8383			
Exploitation hypothesis				
	Symbiotic effectiveness			
Host type	Sympatric	UnH	UnL	*A. heermannii*
Mean nodule mass	$F_{1,244}$ = 0.9239	$F_{1,127}$ = 2.9345	$F_{1,96}$ = 0.0423	$F_{1,127}$ = 3.421
CHR abundance	$F_{1,247}$ = 4.5497*			
SI abundance	$F_{1,237}$ = 4.6492*			

Significance is denoted with asterisks (*P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.0001). Inoculated plants that failed to form nodules were excluded from analyses.

†All *Acmispon* hosts analyzed

CHAPTER 3

Non-nodulating *Bradyrhizobium* modulate the benefits of the legume-rhizobium mutualism

Abstract

Rhizobia are best known for nodulating legume roots and fixing atmospheric nitrogen for the host in exchange for photosynthates. However, the majority and diversity of rhizobia do not form nodules on legumes, often because they lack key loci that are needed to induce nodulation. Non-nodulating rhizobia are robust heterotrophs can persist in bulk soil, thrive in the rhizosphere, or colonize roots as endophytes, but their role in the legume-rhizobium mutualism remains unclear. Here, we investigated the effects of non-nodulating strains on the native *Acmispon-Bradyrhizobium* mutualism. To examine the effects on both host performance and symbiont fitness, we performed clonal inoculations of diverse non-nodulating *Bradyrhizobium* strains on *Acmispon strigosus* hosts and also co-inoculated hosts with mixtures of sympatric nodulating and non-nodulating strains. In isolation, non-nodulating *Bradyrhizobium* strains did not affect plant performance. In most cases, co-inoculation of nodulating and non-nodulating strains reduced host performance when compared to hosts inoculated only with a symbiotic strain. However, co-inoculation increased host performance only under one extreme experimental treatment. Nearly all estimates of nodulating strain fitness were reduced in the presence of non-nodulating strains. We discovered that non-nodulating strains were consistently capable of co-infecting legume nodules in the presence of nodulating strains, but that the fitness effects of co-infection for hosts and symbionts were negligible. Our

data suggest that non-nodulating strains most often attenuate the *Acmispon-Bradyrhizobium* mutualism, and that this occurs via competitive interactions at the root-soil interface as opposed to *in planta*.

Introduction

Rhizobia are heterotrophic soil bacteria with diverse lifestyles. Some rhizobial lineages have acquired the capacity to form nodules on legume roots and fix atmospheric nitrogen for these hosts (Sawada *et al.* 2003). Nodulating rhizobia are attracted to flavonoids released by legumes. In response, the rhizobia secrete nod factors that provoke morphological changes to the roots, enabling the bacteria to enter root cortical cells, become encased by a plant-derived membrane, differentiate into bacteroids, and fix nitrogen (Sprent *et al.* 1987; Lodwig *et al.* 2003). Among nodulating rhizobia, nodulation genes are typically encoded on symbiosis plasmids (Galibert *et al.* 2001; Young *et al.* 2006) or on a genomic island (the 'symbiosis island') (Kaneko *et al.* 2000; Göttfert *et al.* 2001; Kaneko *et al.* 2002; Lee *et al.* 2008). However, soil populations consistently include rhizobia that do not individually nodulate legume hosts (Jarvis *et al.* 1989; Segovia *et al.* 1991; Laguerre *et al.* 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996; Pongsilp *et al.* 2002; Sachs *et al.* 2009; VanInsberghe *et al.* 2015; Hollowell *et al.* 2016), often because they lack key loci that are needed to induce nodulation (Sachs *et al.* 2010a; Okubo *et al.* 2012).

Rhizobial strains that nodulate host roots can have dramatic effects on legume fitness, but these nodulating symbionts must compete with other inhabitants of rhizosphere communities and their relative abundance compared to other microbes can vary (Miethling *et al.* 2000). More specifically, the relative frequency of non-nodulating versus nodulating rhizobia also varies, but non-nodulating genotypes typically dominate and can encompass as much as 99% of the total rhizobial population (Jarvis *et al.* 1989;

Segovia *et al.* 1991; Laguerre *et al.* 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996; Pongsilp *et al.* 2002; Sachs *et al.* 2009; VanInsberghe *et al.* 2015; Hollowell *et al.* 2016). Non-nodulating strains can reduce the number of nodules formed by nodulating strains on legume hosts (Winarno & Lie 1979; Singh & Ahmad 1991) and can invade nodule tissues in the presence of closely related nodulating strains (Pandya *et al.* 2013; Zgadzaj *et al.* 2015). This suggests they may be able to reduce nodulating strain fitness through competitive exclusion at the root surface. Non-nodulating strains have also been shown to promote plant growth on non-legume hosts (Yanni *et al.* 1997; Chaintreuil *et al.* 2000; Yanni *et al.* 2001), but the direct effects of non-nodulating rhizobia on legume host performance remain unclear, either in isolation or when in competition with nodulating strains. Moreover, it is unknown whether non-nodulating rhizobia affect legume host performance while in the rhizosphere or by gaining access to host resources *in planta* as endophytes.

Here, we investigated the effects of non-nodulating strains on native hosts and symbionts of the *Acmispon-Bradyrhizobium* mutualism in California. We inoculated *Acmispon strigosus* hosts (formerly *Lotus strigosus*) with sympatric *Bradyrhizobium* isolates to examine the effects of non-nodulating strains on both host and symbiont performance. Experimental treatments included clonal inoculations with either non-nodulating or nodulating strains, mixed inoculation of nodulating and non-nodulating strains, and inoculation with water as a control. Strain treatments were organized into sixteen unique sympatric co-inoculation strain pairs, with eight pairs each sourced from independent host populations in Northern and Southern California. To investigate factors

that might influence fitness outcomes of inter-strain competition and the ability of non-nodulating strains to co-infect legume nodules, co-inoculation strain pairs varied in terms of genetic relatedness between competing strains and in terms of estimated abundance of each strain in sampled populations (Sachs *et al.* 2009; Hollowell *et al.* 2016). We conducted separate experiments with different co-inoculation ratios. One matched empirical estimates of nodulating versus non-nodulating strain abundance in the *A. strigosus* rhizosphere (Sachs *et al.* 2009; Hollowell *et al.* 2016). The other was extremely biased towards non-nodulating strains to maximize the potential for observing nodule co-infection and the effects of inter-strain competition on modulating the benefits of the legume-rhizobium mutualism. Our goals in these experiments were to examine i) the growth effects of non-nodulating strains on hosts in isolation, ii) the effects of competing non-nodulating strains on host performance and nodulating symbiont fitness, and iii) the genotype specific effects of the nodulating versus non-nodulating strains in determining the fitness outcomes of inter-strain competition.

Materials and Methods

Selection of *Bradyrhizobium* strains and inbred *Acmispon* hosts

Bradyrhizobium isolates were previously collected from the nodules and the soil-root interface of *A. strigosus* host plants at Bodega Marine Reserve (BMR) in Northern California and the University of California Riverside (UCR) in Southern California (Sachs *et al.* 2009; Hollowell *et al.* 2016). All isolates were previously tested for nodulation ability in greenhouse inoculation assays and were genotyped at multiple loci, including genes present on the chromosome (i.e., present in all *Bradyrhizobium*) and genes encoded on the symbiosis island to confirm its presence or absence (Sachs *et al.* 2009; Hollowell *et al.* 2016). Strains for this study were chosen in order to examine the effects of i) field site of origin (BMR vs. UCR), since the sites varied in the relative frequencies of nodulating versus non-nodulating strains (Sachs *et al.* 2009; Hollowell *et al.* 2016), ii) relatedness between competing strains (identity at chromosomal loci vs. unrelated), and iii) strain abundance of each tested strain in their sampled habitat (i.e., rare vs. abundant) (Sachs *et al.* 2009; Hollowell *et al.* 2016). Strains were also selected in order to vary antibiotic resistance profiles, which were used to identify co-infecting strains *in vitro* (Hollowell *et al.* 2015).

From each field site, eight sympatric strains were chosen, composed of three nodulating and five non-nodulating strains, resulting in a total of sixteen *Bradyrhizobium* strains (some strains were used in more than one co-inoculation strain pair). Strains from each field site were grouped into eight sympatric strain pairs to be experimentally co-inoculated, each comprised of one nodulating strain and one non-nodulating strain (**Table**

3.1). Since the primary focus was to investigate the effects of non-nodulating strains, we did not test co-inoculation pairs containing only nodulating or only non-nodulating strains. Antibiotics used to differentiate nodulating and non-nodulating strains within each pair included chloramphenicol (100 μg/ml), carbenicillin (100 μg/ml), gentamicin (100 μg/ml), kanamycin (100 μg/ml), and streptomycin (100 μg/ml). Four of the strain pairs had identical genotypes at two chromosomal loci (recA, glnII) but differed in nodulation ability, modulated by presence or absence of symbiosis island loci (Hollowell *et al.* 2016). The twelve remaining pairs consisted of diverged nodulating and non-nodulating strains that varied in the number of chromosomal SNPs and in local abundance (**Table 3.1**). Results of a pilot study found no evidence of horizontal gene transfer of the symbiosis loci between nodulating and non-nodulating strains; recovered non-nodulating genotypes were re-sequenced to confirm their identity, and PCR on these isolates consistently failed to amplify symbiosis loci (nolL and nodDA), suggesting they did not incorporate the symbiosis island.

Inbred lines of *A. strigosus* were generated from each field site following published protocols (Sachs *et al.* 2009), except that seedlings were transplanted into 1-gallon pots with enriched soil (UCR #3 soil). Plants were grown for 5 months (November 19th 2013 – April 17th 2014) in UCR greenhouse 11 (33.972798, -117.323548) and fruits were picked as they developed (~1500 seeds per plant). No supplemental lighting was used to alter day-length. We chose one inbred line of hosts per site for inoculation (BMR04.02; UCR09.03). All *Bradyrhizobium* strains were inoculated onto sympatric hosts.

Preparation of *Bradyrhizobium* inocula

Each *Bradyrhizobium* strain was initiated from ~2µl of frozen stock and streaked onto plates with Modified Arabinose Gluconate medium (MAG) (Sachs *et al.* 2009). A single colony of each strain was spread onto 5 MAG plates and incubated until lawns formed (29°C, ~8 days). Bacteria were scraped from each plate, resuspended in liquid MAG and concentrations were estimated via optical density (Sachs *et al.* 2010b). The resuspended cells were centrifuged (4,000 rpm, 20 minutes) to remove media and resuspended again in sterile ddH_2O at 10^8 cells ml^{-1}.

Inoculation experiments

Seed preparation and planting followed previously published methods (Sachs *et al.* 2009). Inoculated plants received a total of 5×10^8 rhizobial cells in 5ml of ddH_2O (equivalent by mass to ~10^6 cells g^{-1} soil) which is higher than most estimates of natural rhizobial soil populations (up to 10^5 nodulating cells g^{-1} soil) (Hirsch 1996; Denison & Kiers 2004; Abaidoo *et al.* 2007), but compensates for rhizobial attrition that occurs during the stressful inoculation process (Sachs *et al.* 2009; Sachs *et al.* 2010a; Sachs *et al.* 2010b; Sachs *et al.* 2011; Regus *et al.* 2014; Regus *et al.* 2015; Hollowell *et al.* 2016).

Two separate experiments were conducted with different co-inoculation ratios. The 'ecological experiment' used co-inoculation ratios that matched the empirical population estimates of nodulating versus non-nodulating strain abundance in *A.*

strigosus rhizospheres (i.e., 1:3 at BMR; 1:95, UCR) (Sachs *et al.* 2009; Hollowell *et al.* 2016). The 'extreme experiment' used a co-inoculation ratio of 1:500 nodulating to non-nodulating rhizobia for both host population sources to maximize the potential for competition and nodule co-infection by non-nodulating rhizobia.

For each host population, axenic *A. strigosus* seedlings were separately arranged by size and sized matched seedlings were randomly assigned to sympatric inoculation treatments and greenhouse locations. For each co-inoculation pair, bacterial treatments consisted of i) clonal inoculation of the nodulating strain, ii) clonal inoculation of the non-nodulating strain, iii) co-inoculation of both strains, and iv) inoculation with water (Ecological experiment: 4 treatments per pair × 16 strain pairs × 4 replicate plants per treatment combination × 2 harvest points, 256 plants per host population; Extreme experiment: 4 treatments per pair × 16 strain pairs × 4 replicate plants per treatment combination, 128 plants per host population). Plants were inoculated on 10-3-14 (BMR) and 10-9-14 (UCR).

Harvest and Co-infection analysis

During harvest, plants were removed from the pots and soil was separated from the roots by washing with tap water. Nodules were dissected, counted, and photographed. Roots, shoots, and nodules were separated and oven dried (60°C, >4 days) prior to weighing. We harvested all plants prior to flower formation since this is when nodule senescence in *A. strigosus* often begins in the greenhouse (i.e. around 9 weeks post inoculation, but this can range from 8-24 weeks in the field depending on rainfall) (Sachs

et al. 2010a). Additionally, results of a pilot study suggested co-infection varied with plant developmental stage. To maximize the potential to observe co-infection, half of the plants from each host population in the 'ecological experiment' were harvested 4 weeks after inoculation (n = 128 per host population) and the remaining half at 8 weeks (n = 128 per host population). All plants in the 'extreme experiment' from both host populations were harvested 6 weeks after inoculation (n = 128 per host population). Therefore, at each harvest point, 16 plants per co-inoculation pair (4 plants per inoculation treatment) were harvested.

Frequencies of co-infected nodules and relative proportion of each rhizobial strain within the nodules were estimated for each co-inoculation strain pair at each harvest point. Two (of 4) plants were randomly selected for nodule culturing for each strain pair and harvest week (n = 96). For each sampled plant, 4 randomly selected nodules were chosen for bacterial culturing. We cultured bacteria from a total of 24 nodules per co-inoculation strain, 8 at each harvest week, from 96 test plants (n = 384). The proportion of plants per treatment and the number of nodules per plant selected for culturing was chosen in order to complete harvests in a timely manner and to be consistent with our previous studies (Sachs *et al.* 2010b; Regus *et al.* 2014). Nodules were surface sterilized following previously described methods, crushed, and spread onto 3 MAG plates (Sachs *et al.* 2010b). To estimate relative proportions of the nodulating and non-nodulating strains within the nodule, 100 randomly selected colonies were replica-plated onto MAG plates containing the appropriate antibiotic with plain MAG plates as controls for growth (see **Table 3.1**; detection limit of non-nodulating strains = 1%) (Sachs *et al.* 2010b;

Regus *et al.* 2014). If less than 100 colonies were present, they were all tested for resistance traits.

To confirm that all plated colonies came from the internal portions of the nodules, nodule surface sterilization efficiency was confirmed experimentally. Briefly, 8 *A. strigosus* nodules (collected from 3 separate plants) were dissected from plant roots and each unsterilized nodule was individually rolled over 1 MAG plate using a sterile loop. Nodules were then surfaced sterilized in undiluted bleach (6% sodium hypochlorite) for 2 minutes, rinsed 3 times in sterile water, and subsequently rolled over a second MAG plate to confirm the absence of surface contaminants. Nodules were then crushed using a sterile glass rod and bacteria plated onto a third MAG plate to confirm rhizobial viability within each surface sterilized nodule. Original surface contaminants were present on all nodules tested (i.e. growth on the first MAG plate) and effectively removed in all cases (i.e. no growth on the second MAG plate). Rhizobial viability was confirmed in 7 (of 8) nodules (i.e. growth on the third MAG plate).

Data analysis

We used shoot biomass as our primary estimate of plant performance, which is the most commonly reported plant fitness component (Friesen 2012). We used nodule number and mass as proxies for nodulating strain fitness in our experiments. Previous work by Sachs and colleagues (Sachs *et al.* 2010a) demonstrated that both of these parameters are positively correlated with beneficial rhizobial population sizes in *A.*

strigosus, similar to other systems (Kiers *et al.* 2003; Simms *et al.* 2006; Heath & Tiffin 2007, 2009).

Effects of inoculation treatments on host performance and nodulating strain fitness were analyzed separately for each host population at each harvest week using one-way ANOVAs (d.f. = 3) (Inc. 2012). ANOVAs with significant *F* ratio statistics were followed by pairwise *t*-tests to test for differences among treatments (Inc. 2012). To examine effects of non-nodulating strains on host performance, hosts receiving clonal inoculations of non-nodulating strains were compared to uninoculated controls. To test if competing non-nodulating strains altered host and symbiont fitness during symbiosis, host performance and symbiont fitness were compared between clonal inoculations of nodulating strains and co-inoculation treatments. Net fitness effects were determined by combining plant data from all pairs within an inoculation treatment for each host population and harvest week (n = 128). Effects within each pair were analyzed using plants only from each respective pair for each host population and harvest week (n = 16).

Nodules and plants were scored as co-infected if ≥1 replica-plated colonies were identified as a non-nodulating strain. Although several factors can influence the ability of a non-nodulating strain to co-infect legume nodules, the primary objectives in our co-infection analyses were to i) estimate the co-infection ability of each non-nodulating strain, ii) detect any patterns in co-infection ability, and iii) determine if there are any host performance costs to co-infection. Thus, co-infection ability for each non-nodulating strain was assessed using data from all harvest weeks and co-inoculation ratios where each non-nodulating strain was present in the inoculum. Since half of the co-inoculated

plants were selected for culturing and bacteria from 4 nodules per plant were replica plated, we regard our estimates of co-infection as conservative.

Strain variation in the capacity to co-infect nodules or block co-infection was analyzed using one-way ANOVAs followed by Tukey's HSD test among non-nodulating and nodulating strains, respectively. To test if the proportion of co-infection for chromosomally identical pairs differed from chromosomally diverged pairs (0, >10 SNPs) we used a generalized linear mixed model with genetic divergence (identical or diverged) as a fixed effect and co-inoculation strain pair as a random effect (Fit Model Platform in JMP 10.0). To examine if co-infection frequency scales with genetic distance, a correlation analysis was performed between the proportion of nodules co-infected per plant and the number of SNPs between each co-inoculation strain pair.

To examine the effects of co-infection, host performance and symbiont fitness were compared using one-way ANOVAs between co-inoculated plants without evidence of co-infection and co-infected plants (where nodule sub-culturing data were available) separately for each host population and harvest week (d.f. = 1; n = 16).

Results

Effects of non-nodulating strains on hosts in isolation

None of the non-nodulating strains formed nodules or any detectable features on roots when inoculated in isolation (**S3.1**). In no case did clonal inoculation with a non-nodulating strain affect host growth compared to uninoculated control plants (**Table 3.2**).

Effects of competing non-nodulating strains on host growth and symbiont fitness

In the ecological experiment, co-inoculation of nodulating and non-nodulating strains reduced host growth and measures of nodulating strain fitness in both host populations. For the BMR population, the co-inoculated treatments reduced net host growth by ~15% and net nodulating strain fitness was significantly decreased in terms of total nodule number (~18% reduction), but net total nodule biomass was not significantly affected (**Figure 3.1a; Table 3.2**). Nodulating strain fitness proxies, but not host growth, varied based on strain combinations for BMR hosts (**Table 3.2**). For the UCR population, net host performance was significantly reduced by ~28% in co-inoculated treatments. Net nodulating strain fitness was also decreased in co-inoculated treatments at UCR, with a ~29% and ~32% reduction in total nodule number and total nodule biomass, respectively (**Figure 3.1a; Table 3.2**). Host performance and nodulating strain fitness proxies varied based on strain combinations for UCR hosts (**Table 3.2**). The mean number of nodules per plant, per strain pair, per harvest week is reported in the supplemental materials (**S3.2**).

Extreme co-inoculation ratios of nodulating to non-nodulating strains resulted in varying effects for each host population. Net BMR host growth was significantly increased by ~24% under extreme co-inoculation conditions compared to clonally inoculated plants, yet net total nodule number was reduced by ~19% (**Figure 3.1b; Table 3.2**). Similar to inoculation with an ecologically relevant ratio, net total nodule biomass was not significantly different among treatments (**Table 3.2**). All metrics of net host growth and nodulating strain fitness were significantly decreased in UCR hosts (**Figure 3.1b; Table 3.2**). Co-inoculation with an extreme ratio of non-nodulating strains resulted in a ~17% reduction in net host growth, a ~21% reduction in net total number of nodules, and a ~23% reduction in net total nodule biomass (**Figure 3.1b; Table 3.2**). Host growth and nodulating strain fitness proxies varied based on strain combinations for both host populations under an extreme co-inoculation ratio (**Table 3.2**).

Effects of non-nodulating strain co-infection on host and symbiont fitness

Data were analyzed from 296 nodules from which nodule occupancy was successfully estimated by sub-culturing (**Table 3.1**; **S3.1**). Co-infection ability and estimated within nodule population proportion of each non-nodulating strain was determined using data from all harvest weeks and co-inoculation ratios. All non-nodulating strains were able to colonize the nodule tissue of at least one *A. strigosus* nodule and evidence of co-infection was uncovered in all tested co-inoculation pair combinations, except for pairs 6, 8, and 15 (**Figure 3.2; Figure 3.3a**). Non-nodulating strains exhibited variation in both their ability to co-infect *A. strigosus* nodules and their

within nodule population estimates (**Figure 3.3a and b**). However, the capacity to co-infect nodules was not correlated with strain genotype abundance (R^2 = 0.0015, P = 0.7081).

Non-nodulating strains that were paired with genetically identical nodulating strains (strains 15, 80, 98, and 110) co-infected significantly more nodules per plant, compared to non-nodulating strains in genetically diverged co-inoculation pairs (generalized linear mixed model: $F_{1,96}$ = 6.21, P = 0.0258 and SNP correlation analysis R^2 = -0.0719, P = 0.0086, n = 96; **Figure 3.2; Figure 3.3a**). However, we did not find any significant differences in terms of estimated within nodule population (**Figure 3.3b)**. None of the nodulating strains were able to prevent co-infection and the proportion of co-infected nodules per plant was not significantly different among nodulating strains (**Figure 3.3c**).

No significant effects of co-infection were found on host or nodulating strain fitness in either host population in the ecological and extreme ratio experiments (**Table 3.3**). Co-infection is reduced over time in the ecological experiment in both host populations (harvested at 4 and 8 weeks), although this trend is not significant for BMR host plants (**Figure 3.4**).

Discussion

Rhizobia are increasingly understood to have multifarious lifestyles, including root-nodule symbiosis, colonization of plant roots in the rhizosphere or as root endophytes, and independent growth in the soil or other habitats (Yanni *et al.* 1997; Chaintreuil *et al.* 2000; Yanni *et al.* 2001; Denison & Kiers 2004; Pandya *et al.* 2013; Zgadzaj *et al.* 2015). Yet, these lifestyles can be transient and are only partially dependent on the presence or absence of symbiosis loci, which have been a major focus of research. Nodulating strains with the canonical nodulation loci, for instance, are not capable of forming nodules on all hosts (e.g., host specificity (Turk & Keyser 1992; Mpepereki *et al.* 1996; Sachs *et al.* 2009)). Furthermore, non-nodulating strains lacking key nodulation genes can co-infect legume nodules, expropriating a symbiotic role (Pandya *et al.* 2013; Zgadzaj *et al.* 2015). Regardless of what factors determine lifestyle, strains that do not form nodules comprise the majority of sampled rhizobial populations (Jarvis *et al.* 1989; Segovia *et al.* 1991; Laguerre *et al.* 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996; Pongsilp *et al.* 2002; Sachs *et al.* 2009; VanInsberghe *et al.* 2015; Hollowell *et al.* 2016). Our data here suggest that non-nodulating rhizobia are not passive players in the host rhizosphere. Instead, non-nodulating rhizobia can have considerable effects on the legume-rhizobium symbiosis, most often by reducing plant performance and attenuating nodulating symbiont fitness.

Our results from clonal inoculations corroborate previous work, which found no effect of non-nodulating strains on legume host growth in isolation (Sachs *et al.* 2009). However, under parameters that model relative abundances of non-nodulating strains within the rhizosphere, our dataset revealed substantial costs to host growth and symbiont

fitness in both host populations examined (except for total nodule biomass at BMR; **Figure 3.1a; Table 3.2**). Our data are consistent with previous reports of a reduction in nodulating strain fitness, measured by number of nodules formed by nodulating strains (Winarno & Lie 1979; Singh & Ahmad 1991), but reveal that this does not always result in a significant decrease in all rhizobial fitness estimates (i.e. total nodule biomass). The higher ratio of non-nodulating strains in ecologically relevant UCR co-inocula could explain this difference and further suggests a competitive role for non-nodulating strains at the root-soil interface, but we are unable to disentangle the effects of co-inoculation ratio from any differences due to host genotype.

Host growth response to extreme ratios of non-nodulating strains differed between host populations. Co-inoculation resulted in a significant increase in net BMR host growth (although this trend was only significant for strain pair #8; **Table 3.2**), yet reduction in nodule number and effect on total biomass were similar compared to ecologically relevant ratios (**Figure 3.1a and b; Table 3.2**). Legumes have finely tuned mechanisms to regulate nodule number (Ferguson *et al.* 2010), but the number of nodules formed in any interaction is nonetheless a product of the host and rhizobium genotype (Heath & Tiffin 2007; Porter & Simms 2014). This suggests that host control over nodule number is incomplete (Regus *et al.* 2015). Just as ineffective rhizobia form many nodules on hosts without benefiting the host (Sachs *et al.* 2010b), effective rhizobium strains might often produce more nodules on a host than is optimal for host growth. Thus, the reduction of nodule number by non-nodulating strains could actually increase host growth if the nodulation strains present are prolific nodule producers. However, this

hypothesis cannot be explicitly tested with our data since there was no significant variation in nodule number for BMR populations. All estimates of host performance and nodulating strain fitness were reduced under extreme co-inoculation conditions at UCR (**Figure 3.1b; Table 3.2**), although they were not as pronounced when compared to co-inoculations with ecologically relevant ratios. Differences in host growth among treatments are more distinct as plants approach flowering (closer to the harvest at 8 weeks in the ecological experiment), thus the magnitude in the reduction in host performance and nodulating strain fitness might have been more comparable if the extreme experiment was harvested at a later date.

Legume nodules can harbor multiple lineages of bacteria (Philipson & Blair 1957; Sturz *et al.* 1997; de Lajudie *et al.* 1999; Tokala *et al.* 2002; Bai *et al.* 2003; Mhamdi *et al.* 2005; Mrabet *et al.* 2006; Zakhia *et al.* 2006; Muresu *et al.* 2008), yet few studies have considered the capacity of rhizobial strains lacking nodulation loci to invade and persist within nodule tissue (but see (Pandya *et al.* 2013; Zgadzaj *et al.* 2015)). Ours is the first study to explore the potential for co-infection using native combinations of strains on sympatric hosts. All the non-nodulating strains that we tested were able to co-infect nodules (**Figure 3.3a and b**) and this was true in nearly every tested strain combination (**Figure 3.2**). This suggests co-infection with non-nodulating strains is likely to be at least as common as co-infection with non-rhizobial bacteria. These data lend support to past reports of rhizobia that were isolated from legume nodules, but were subsequently found to be unable to form nodules in inoculation tests (Rangin *et al.* 2008; Wu *et al.* 2011). Our data also imply that co-infection ability can vary depending on both

rhizobial and plant factors. Firstly, the estimated natural abundance of non-nodulating strains did not appear to impact co-infection ability, but genetic relatedness between strains did have a significant effect. The proportion of co-infected nodules was increased in strain pairs that were more closely related (0 SNPs; **Table 3.1**), compared to strain pairs that were more distantly related (>10 SNPs; **Table 3.1**). One explanation for this result could be the similarity of critical signaling molecules during root colonization are more important than abundance for non-nodulating strains (e.g. exopolysaccharides or EPS) (Zgadzaj *et al.* 2015). Recently Zgadzaj and colleagues (2015) found symbiotic rhizobia with compatible EPS had an advantage over co-infecting endophytes with different EPS molecules. In *Bradyrhizobium,* EPS genes are chromosomally encoded (not within the symbiosis island) (Kaneko *et al.* 2002). Hence, it is possible that chromosomally identical (i.e. 0 SNPs) co-inoculation pairs make similar, if not identical EPS, explaining the higher co-infection rates compared to strain pairs that are genetically unrelated (i.e. >10 SNPs). Evidence of co-infection decreased over time since inoculation (**Figure 3.4**). Previous work has shown legume hosts can actively sanction ineffective rhizobial strains (non-fixing), reducing nodule growth rate and within nodule rhizobial population sizes (Denison 2000; Kiers *et al.* 2003; Sachs *et al.* 2010b; Regus *et al.* 2014). Since the presence of non-nodulating strains within legume nodules did not increase host growth in this study (**Table 3.3**), we can consider non-nodulating strains to be similar to ineffective infections. Sanctions mechanisms could be one reason for the observed decline of co-infection over time, but we are unable to discern if the host is controlling non-nodulating strain population sizes via sanctions or if non-nodulating strains are

poorly adapted to survival and proliferation within the nodule environment. Lastly, while co-infection is prevalent, the lack of any measureable effects on host growth suggests that it might not play a critical role in terms of host fitness (**Table 3.3**).

Theoretical and empirical work on the legume-rhizobium symbiosis has generally assumed that legume fitness is predominately regulated by which rhizobial strains successfully nodulate host roots (Triplett & Sadowsky 1992; Kiers *et al.* 2003; Denison & Kiers 2004; Sachs *et al.* 2010a; Sachs *et al.* 2010b; Schumpp & Deakin 2010; Yates *et al.* 2011; Regus *et al.* 2014). Investigations have sought to uncover the mechanisms of competition among nodulating strains of varying symbiotic effectiveness (i.e., nitrogen fixation capacity), and to understand how the outcomes of this competition affect host fitness. Although effective nodulating strains can be competitive for nodulation (Yates *et al.* 2005; Yates *et al.* 2008; Friesen 2012), competitive ability may not be correlated with beneficial quality (Bloem & Law ; Hafeez *et al.* ; Vásquez-Arroyo *et al.* ; Triplett & Sadowsky 1992; Simms *et al.* 2006). Researchers attempting to apply highly effective rhizobial strains to improve legume crop commonly find that these strains nodulate hosts at low rates, and that the inoculant strains get outcompeted by less efficient symbionts (Triplett & Sadowsky 1992; Den Herder & Parniske 2009; Schumpp & Deakin 2010; Yates *et al.* 2011). Despite the prevalence and dominance of non-nodulating strains (Jarvis *et al.* 1989; Segovia *et al.* 1991; Laguerre *et al.* 1993; Sullivan *et al.* 1995; Sullivan *et al.* 1996; Pongsilp *et al.* 2002; Sachs *et al.* 2009; VanInsberghe *et al.* 2015; Hollowell *et al.* 2016), such studies have neglected the impact of endemic non-nodulating strains as potential negative competitors on the mutualism. Our work illustrates endemic

non-nodulating rhizobial strains often co-infect legume nodules and, more importantly, may play an active role in modulating the legume-rhizobium mutualism. Our results also show non-nodulating rhizobia lack effects on host growth in isolation and during nodule co-infection, suggesting that the key fitness effects of non-nodulating strains are mediated by inter-strain competition at the root-soil interface before nodulation occurs. Further research is necessary to understand the specific mechanisms of inter-strain competition within the microbiota of the rhizosphere, but the overall competitive effects of non-nodulating rhizobial strains and other non-nodulating rhizosphere microbes should be promptly considered both in bioinoculant development and in research.

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Figure 3.1 Co-inoculation alters host growth and symbiont fitness. Shoot biomass and total nodule number data was merged within treatment from all pairs from the same host population. (A) Shoot biomass and total nodule number from each host population in the Ecological ratio experiment 8 weeks post inoculation. (B) Shoot biomass and total nodule number from each host population in the Extreme ratio experiment 6 weeks post inoculation. Asterisks denote significant differences between net clonal inoculations of nodulating strains and net co-inoculations with non-nodulating strains within each host population. (One-way ANOVAs: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). Error bars represent 1 standard error.

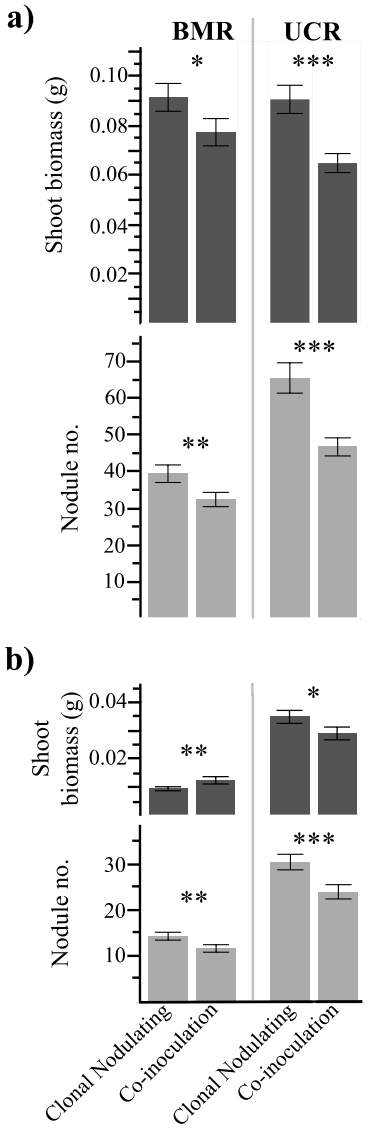

Figure 3.2 Proportion of co-infected nodules by strain combination. Co-infection proportions for each strain pair were averaged across all harvest weeks and inoculation ratios within each source host population. Stars represent co-inoculation pairs where strains are genetically identical at the chromosomal level. Error bars represent 1 standard error.

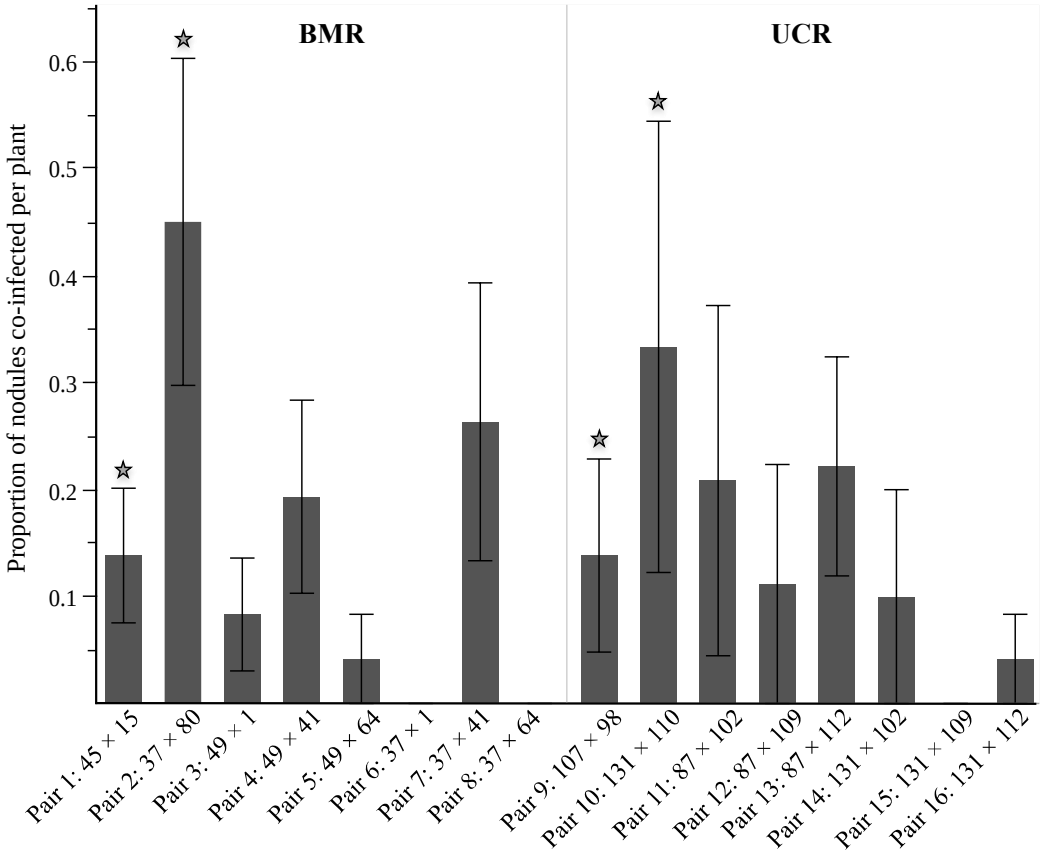

Figure 3.3 Proportion of co-infected nodules by individual strain and within nodule proportion estimates. Co-infection proportions were averaged for each strain using data from all strain combinations. Stars represent non-nodulating strains that are genetically identical to co-inoculated nodulating strains at the chromosomal level. (a) Proportion of co-infected nodules for each non-nodulating strain. Letters are significant differences among non-nodulating strains (Tukey's HSD test, $p < 0.05$). (b) Mean proportion of each non-nodulating strain within the total nodule population per plant. (c) Proportion of co-infected nodules for each nodulating strain. Error bars represent 1 standard error.

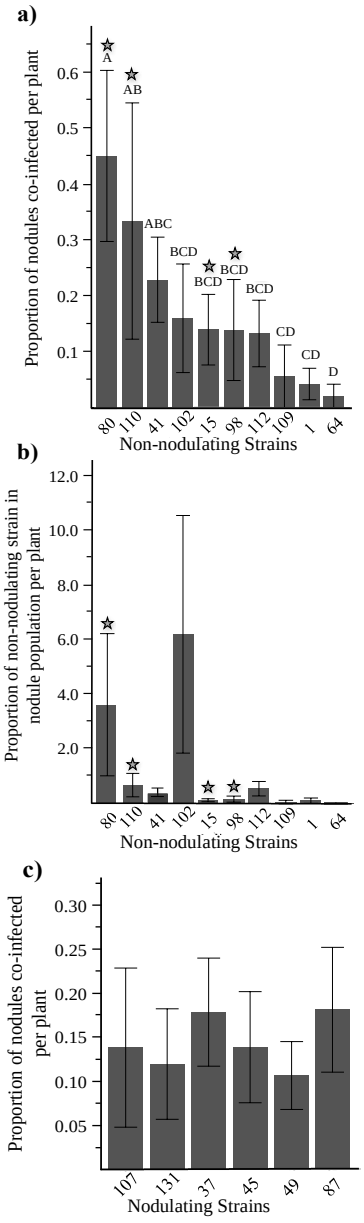

Figure 3.4 Proportion of co-infection in the Ecological ratio experiment at 4 and 8 weeks post inoculation for each host population. Proportion of nodules co-infected per plant was calculated by averaging all co-inoculated treatment plants from all pairs in the same host population (one-way ANOVA: **, P < 0.01). Error bars represent 1 standard error.

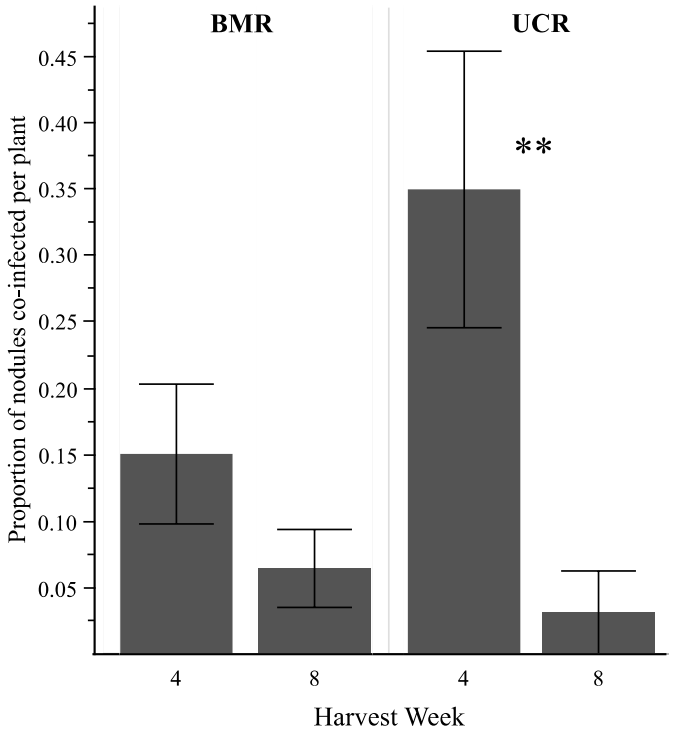

Table 3.1 Summary of strain features and antibiotic resistance. Number of SNPs between strains and genotype abundance was previously determined by Hollowell and colleagues (2016a). Antibiotic abbreviations are as follows: CHL, chloramphenicol; GEN, gentamicin, STR, streptomycin; CAR carbenicillin; KAN, kanamycin.

	Pair no.	No. of SNPs between strains	Strain	Genotype	Genotype Abundance	Nodulation Ability	Antibiotic Resistance
BMR							
	1	0	45	G01_R01	24	+	CHL^r
			15			-	CHL^s
	2	0	37	G05_R02	24	+	CHL^s
			80			-	CHL^r
	3	83	49	G03_R01	355	+	GEN^r
			1	G17_R17	8	-	GEN^s
	4	40	49	G03_R01	355	+	GEN^r
			41	G112_R09	1	-	GEN^s
	5	58	49	G03_R01	355	+	STR^s
			64	G16_R16	2	-	STR^r
	6	64	37	G05_R02	24	+	GEN^r
			1	G17_R17	8	-	GEN^s
	7	17	37	G05_R02	24	+	GEN^r
			41	G112_R09	1	-	GEN^s
	8	48	37	G05_R02	24	+	STR^s
			64	G16_R16	2	-	STR^r
UCR							
	9	0	107	G03_R01	355	+	GEN^r
			98			-	GEN^s
	10	0	131	G11_R07	62	+	CAR^r
			110			-	CAR^s
	11	73	87	G03_R01	355	+	KAN^s
			102	G36_R35	20	-	KAN^r
	12	108	87	G03_R01	355	+	GEN^r
			109	G42_R47	5	-	GEN^s
	13	12	87	G03_R01	355	+	CAR^r
			112	G04_R21	1	-	CAR^s
	14	77	131	G11_R07	62	+	KAN^s
			102	G36_R35	20	-	KAN^r
	15	110	131	G11_R07	62	+	GEN^r
			109	G42_R47	5	-	GEN^s
	16	12	131	G11_R07	62	+	GEN^r
			112	G04_R21	1	-	GEN^s

Table 3.2 Effects of non-nodulating strains in altering host and symbiont fitness. *F* ratio statistic reported from one-way ANOVAs comparing effects of inoculation treatments (d.f. = 3) by harvest week, net source host population (n = 128) and within co-inoculation pair (n = 16). Asterisks indicate significant *F* ratio statistics (**P* < 0.05, ***P* < 0.01, ****P* < 0.0001). To determine significant differences among inoculation treatments, indicated in boldface type, ANOVAs were followed by pairwise *t*-testes comparing inoculation treatments (*P* < 0.05).

	Ecological Ratio Week 8			Extreme Ratio Week 6		
	F - Shoot biomass	*F* - Nodule no.	*F* - Total nodule biomass	*F* - Shoot biomass	*F* - Nodule no.	*F* - Total nodule biomass
BMR						
Net[+]	**119.12***b**	**185.36***b**	69.36***	**44.73***c**	**164.25***b**	90.50***
Pair 1	20.52***	**32.76***b**	**49.79***b**	3.67*	15.09**	4.40*
Pair 2	12.67**	13.72**	6.46**	3.16	**15.87**b**	**9.94**b**
Pair 3	4.60*	16.79***	9.71**	2.41	11.69**	32.78***
Pair 4	38.16***	22.72***	31.91***	10.64**	20.28***	11.86**
Pair 5	25.34***	18.72***	18.23***	2.49	33.81***	14.10**
Pair 6	12.78**	15.79**	14.16**	6.69**	34.16***	8.97**
Pair 7	43.93***	43.50***	29.75***	9.13**	**27.94***b**	15.20**
Pair 8	12.95**	13.26**	4.33*	**8.14**c**	19.91***	18.68***
UCR						
Net[#]	**130.82***b**	**192.01***b**	**140.82***b**	**77.23***b**	**191.68***b**	**62.75***b**
Pair 9	**29.34***b**	**267.68***b**	**32.22***b**	12.82**	13.50**	5.91*
Pair 10	42.92***	28.26***	144.57***	8.27**	34.76***	10.94**
Pair 11	**18.27***b**	18.87***	29.10***	13.67**	**95.90***b**	9.46**
Pair 12	**15.64***b**	**22.29***b**	**10.67**b**	8.39**	13.79**	12.84**
Pair 13	**17.38***b**	**64.27***b**	**18.78***b**	7.29**	**14.01**b**	6.17**
Pair 14	23.98***	**52.08***b**	**33.12***b**	7.86**	**87.32***b**	12.34**
Pair 15	6.52**	13.36**	10.25**	**26.54***b**	**37.96***b**	**56.20***b**
Pair 16	6.06**	6.23**	5.45*	**19.04***b**	18.37***	8.819**

[+]Refers to plants from all pairs sourced from BMR merged within treatment
[#]Refers to plants from all pairs sourced from UCR merged within treatment
a Refers to cases where uninoculated control treatments differ significantly from clonal non-nodulating treatments
b Refers to cases where clonal nodulating treatments are significantly *higher* than co-inoculation treatments
c Refers to cases where clonal nodulating treatments are significantly *lower* than co-inoculation treatments

Table 3.3 Effects of co-infection on host and symbiont fitness. *F* ratio statistic reported from one-way ANOVAs comparing effects of co-infection (d.f. = 1) by harvest week and net source host population (n = 16). Asterisks indicate significant *F* ratio statistics (*P < 0.05, **P < 0.01, ***P < 0.0001).

		Harvest Week	*F* - Shoot biomass (g)	*F* - Root biomass (g)	*F* - Nodule no.	*F* - Total nodule biomass (g)
Ecological Ratio						
	BMR[+]					
		4	0.001	0.182	0.187	0.568
		8	0.005	0.090	0.404	1.341
	UCR[#]					
		4	0.020	0.066	0.021	0.845
		8	0.223	0.039	2.505	0.302
Extreme Ratio						
	BMR[+]					
		6	4.090	0.656	0.024	1.280
	UCR[#]					
		6	0.192	0.385	0.301	1.553

[+]Refers to plants from all pairs sourced from BMR merged within treatment

[#]Refers to plants from all pairs sourced from UCR merged within treatment

GENERAL CONCLUSION

In the legume-rhizobium mutualism, hosts can selectively favor beneficial symbionts over ineffective genotypes (Singleton & Stockinger 1983; Denison 2000; Simms & Taylor 2002; Kiers *et al.* 2003; Simms *et al.* 2006; Sachs *et al.* 2010; Oono *et al.* 2011; Regus *et al.* 2014), but ineffective rhizobia persist. In the first chapter, I found that symbiotic function in *Bradyrhizobium* is evolutionarily unstable and that variation in symbiotic quality is likely driven by mechanisms acting at the population level, as opposed to among populations. In the second chapter, I tested if symbiotic ineffectiveness was a context dependent, maladapted outcome. Instead, I found that some ineffective *Bradyrhizobium* can overcome host sanctioning mechansims and attain relatively high fitness in the soil. These data suggest host exploitation could also maintain ineffective rhizobia in natural populations. In the last chapter, I tested if non-symbiotic conspecifics could modulate the benefits provided during symbiosis. I found that not only do non-nodulating *Bradyrhizobium* most often reduce host performance and nodulating rhizobial fitness via competitive interactions at the root surface, but they also coinfect legume nodules and expropriate a symbiotic role.

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