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LETTER

Interspecific conflict and the evolution of ineffective rhizobia

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Abstract

Microbial symbionts exhibit broad genotypic variation in their fitness effects on hosts, leaving hosts vulnerable to costly partnerships. Interspecific conflict and partner-maladaptation are frameworks to explain this variation, with different implications for mutualism stability. We investigated the mutualist service of nitrogen fixation in a metapopulation of root-nodule forming *Bradyrhizobium* symbionts in *Acmispon* hosts. We uncovered *Bradyrhizobium* genotypes that provide negligible mutualist services to hosts and had superior *in planta* fitness during clonal infections, consistent with cheater strains that destabilise mutualisms. Interspecific conflict was also confirmed at the metapopulation level – by a significant negative association between the fitness benefits provided by *Bradyrhizobium* genotypes and their local genotype frequencies – indicating that selection favours cheating rhizobia. Legumes have mechanisms to defend against rhizobia that fail to fix sufficient nitrogen, but these data support predictions that rhizobia can subvert plant defenses and evolve to exploit hosts.

Keywords

Acmispon strigosus, *Bradyrhizobium*, cheating, legume–rhizobium mutualism, maladaptation, mutualism breakdown, sanctions, symbiosis.

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INTRODUCTION

Biologists since Darwin have struggled to understand the evolutionary maintenance of mutualism (Darwin 1859). Natural selection is understood to shape traits that optimise individual fitness (Williams 1966), so cheaters that gain from the cooperation of others – but do not reciprocate – can arise and destabilise mutualisms (Sachs & Simms 2006; Sachs *et al*. 2011b). However, empirical evidence for cheaters has been scant (Jones *et al*. 2015), causing biologists to question the ecological relevance of cheating (Friesen 2012; Frederickson 2013). A challenge in investigating cheating is distinguishing cheaters from partners that fail to cooperate for non-adaptive reasons, which can include mutations in mutualism genes or associating with mismatched partners (Sachs 2015). Another complication is that cheating might vary ecologically in its occurrence (Bull & Rice 1991; West *et al*. 2002b), depending on the presence or type of partner defenses (Foster & Kokko 2006; Steidinger & Bever 2014, 2016) or extrinsic resources that are traded by mutualists (Kiers *et al*. 2007; Regus *et al*. 2014; Weese *et al*. 2015; Wendlandt *et al*. 2019). Fundamental questions about cheating remain unanswered, in particular whether cheaters are common in natural populations, and whether particular ecological settings promote cheating.

The legume–rhizobia mutualism is an excellent system in which to investigate cheating because it is easy to measure the benefits rhizobia provide and obtain from their hosts. Rhizobia are free-living soil bacteria that can instigate the formation of nodules on legume roots and fix nitrogen for their hosts in exchange for photosynthates (Sprent *et al*. 1987). Nitrogen fixation by rhizobia enhances growth of diverse legumes, generates much of the fixed nitrogen for our biosphere, and accelerates ecosystem development in nutrient poor habitats (Vitousek *et al*. 1997). However, rhizobia genotypes vary greatly in nitrogen fixation (Sachs *et al*. 2018) and genotypes that fix little or no nitrogen, known as ineffective rhizobia, exist in natural and agricultural soils (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). Legumes have mechanisms that defend against ineffective rhizobia (Sachs *et al*. 2018). Firstly, legumes can regulate nodule formation and growth dependent on the net benefits gained from nodulation (Regus *et al*. 2015; Quides *et al*. 2017; Wendlandt *et al*. 2019). Moreover, legumes can 'sanction' rhizobia by arresting *in planta* proliferation of ineffective strains (Singleton & Stockinger 1983; Kiers *et al*. 2003; Simms *et al*. 2006; Sachs *et al*. 2010b; Oono *et al*. 2011; Regus *et al*. 2014, 2017a). Such host mechanisms should select against rhizobia that fix little or no nitrogen (Denison 2000; Simms & Taylor 2002; West *et al*. 2002a,b). Thus, the widespread persistence of ineffective rhizobia represents a dilemma with impacts on crops and wild plant communities.

Two overarching frameworks model the persistence of ineffective rhizobia, here described as partner-maladaptation and interspecific conflict. In the partner-maladaptation model, ineffective rhizobia exhibit inferior fitness compared to

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beneficial rhizobia and persist only by recurrent mutations or by introductions onto mismatched hosts. If mutations in symbiosis loci spontaneously generate ineffective rhizobia, they could be maintained via mutation-selection balance, wherein ineffective mutants are introduced into populations and persist until they are purged by host defense (Van Dyken *et al*. 2011). In parallel, rhizobia strains can encounter hosts upon which they are conditionally ineffective, because the rhizobia are adapted to other hosts or to free-living conditions in the soil (Burdon *et al*. 1999; Heath & Tiffin 2007; Heath 2010; Sachs *et al*. 2011a). In the interspecific conflict model, rhizobia evolve adaptively to exploit hosts, either by manipulating the plant to extract additional benefits or by escaping host defense. In this model ineffective rhizobia save costs by not fixing nitrogen and experience superior fitness relative to other genotypes; i.e. 'cheater mutants' (West *et al*. 2002b; Ghoul *et al*. 2014; Jones *et al*. 2015; Sachs 2015). These frameworks are not mutually exclusive; maladapted and cheating strains might coexist in populations and we can also consider models that expand beyond these frameworks. For instance, nutrient-rich soils provide legumes with an inexpensive source of nitrogen, which might cause hosts to downregulate defense pathways (in the short term) or favour hosts with relaxed defenses (over evolutionary time), either of which might promote ineffective rhizobia (Kiers *et al*. 2006, 2007; Weese *et al*. 2015; Wendlandt *et al*. 2019). The capacity of hosts to invest in rhizobia or impose defenses could also vary spatiotemporally depending on the metabolic costs of these host traits weighed against the local frequency of ineffective rhizobia (Foster & Kokko 2006; Steidinger & Bever 2014, 2016). Finally, the degree of partner specificity of hosts or symbionts could vary. Partner generalism can be favoured over specialisation when a diversity of mutualist partners is available (Batstone *et al*. 2018) which can also favour the evolution of less beneficial partners (Ehinger *et al*. 2014).

Here, we investigate *Bradyrhizobium* spp. associated with *Acmispon strigosus* (formerly *Lotus strigosus*), an annual legume native to the southwestern USA. *Bradyrhizobium* isolates were collected from six *A. strigosus* populations across California that exhibit diverged *Bradyrhizobium* communities (Hollowell *et al*. 2016a,b) and experience ~ 10-fold variation in soil nitrogen concentrations, driven by anthropogenic nitrogen deposition over the last 70+ years (Regus *et al*. 2014, 2017b; Wendlandt *et al*. 2019). All tested *A. strigosus* lines arrest *in planta* proliferation of ineffective rhizobia (i.e. sanctions), but the host lines exhibit segregating variation in their regulation of nodule growth, with some plant lines adjusting nodule size dependent on the symbiotic quality of the rhizobia present (i.e. scaled investment) and other lines exhibiting fixed nodule size (i.e. unscaled investment; (Wendlandt *et al*. 2019). We analysed symbiotic effectiveness and fitness proxies for thirty phylogenetically diverse isolates of *Bradyrhizobium*. We performed clonal inoculations of each *Bradyrhizobium* genotype onto five *Acmispon* host types, comprising two *A. strigosus* plant lines from the same sampling location as the rhizobia (i.e. sympatric lines), two *A. strigosus* lines that were universally used for all strains (i.e. universal lines, mostly allopatric combinations), and an outbred seed set of *A. heermannii*, a sister taxon from an allopatric source (Allan & Porter 2000). Our study investigated three questions. Firstly, we asked how common ineffective *Bradyrhizobium* are in natural populations to address their ecological relevance. Secondly, we examined whether *Bradyrhizobium* effectiveness varies depending on host genotype or local soil nitrogen to provide insight into evolutionary mechanisms that can maintain variation in mutualist services. Finally, we investigated whether rhizobia fitness correlates with symbiotic effectiveness to test the partner-maladaptation and interspecific conflict models.

MATERIALS AND METHODS

Collection and genotyping of *Bradyrhizobium* isolates

Bradyrhizobium were isolated from the nodules and the soil–root interface of *A. strigosus* plants from six populations across California between 2005 and 2013, with most populations sampled in multiple years (Hollowell *et al*. 2016a). Populations were chosen based on long-term differences in soil nitrogen concentrations, with three populations experiencing relatively high concentrations (0.04–0.11% total nitrogen; Griffith Park, GRI; Bernard Biological Field Station of the Claremont Colleges, CLA; University of California Riverside, UCR) and three others having lower concentrations (0.01–0.03% total nitrogen; Bodega Marine Reserve, BMR; Burns Piñon Ridge Reserve near Yucca Valley, YUC; Anza Borrego Desert State Park, ANZ) (Regus *et al*. 2014, 2017b). Previous work using nitrogen supplementation suggested that nitrogen-rich soils favour the evolution of ineffective rhizobia (Weese *et al*. 2015).

From 72 plants, 5–26 nodules were sampled and from each nodule a single clone of *Bradyrhizobium* was isolated by surface sterilising the nodule, plating its contents on solid media, and genotyping a single colony (Sachs *et al*. 2009). Root surface isolates were also taken by washing dissected root sections in sterile ddH_20 and plating on multiple selective media; and were only included among the test isolates if they showed evidence of nodulation ability (Sachs *et al*. 2009; Hollowell *et al*. 2016a). From the six populations, a total of 444 isolates were previously assigned to genotypes based on concatenation of two chromosomal locus sequences (i.e. CHR genotype; *glnII*, *recA*) (Hollowell *et al*. 2016a). A subset of 255 isolates were genotyped for two loci on the *Bradyrhizobium* symbiosis island (i.e. SI genotype; *nodZ*, *nolL*; (Hollowell *et al*. 2016b)).

Genotype frequencies were estimated for each isolate as a proxy of population-level fitness, since selective sweeps cause some genotypes to reach high frequencies (Sullivan *et al*. 1995; Sullivan & Ronson 1998; Epstein *et al*. 2012; Hollowell *et al*. 2016b). Phylogenetic relationships among isolates were reconstructed using the CHR and SI loci with PhyML 3.0 utilising a BioNJ starting tree and subtree pruning and regrafting (Guindon *et al*. 2010). The CHR and SI genome regions were analysed separately because the SI can be horizontally transferred (Hollowell *et al*. 2016b). For each isolate and for each genome region, genotype frequency was calculated as the proportion of isolates from the sampled population that were identified as the focal genotype (Hollowell *et al*. 2016a). Within each population, we attempted to choose at least one isolate per quartile of genotype frequency. Four

to six isolates were selected from each field site (thirty isolates total), to be analysed for symbiotic effectiveness and fitness parameters. Isolates without previously assigned SI genotypes (#'s 137, 138, 139, 141, 147, 149 and 152) were initially chosen based on CHR genotype frequency (Table S1). For isolates with missing SI data, DNA was sequenced at UC Riverside. GenBank accessions are published elsewhere (Hollowell *et al.* 2016a,b) or listed below (Table S2).

Acmispon hosts

We quantified effectiveness and fitness of rhizobia on locally encountered hosts (sympatric experiment) and separately tested if these traits vary dependent on host genotype (universal experiment). Given the number of isolates tested, a single full-factorial design was not practical. *A. strigosus* and *A. heermannii* are closely related (Allan & Porter 2000), broadly sympatric (www.calflora.org), grow in similar habitats of semi-open patches of sand, gravel, and cryptogamic crusts and share similar communities of *Bradyrhizobium* (Sachs *et al.* 2009). Fourteen lines of *A. strigosus* and outbred seeds of *A. heermannii* were used for inoculation experiments. *A. strigosus* hosts included two lines originating from each of the six populations to be inoculated with *Bradyrhizobium* isolates from the same source location (i.e. sympatric combinations of hosts and strains). Two additional *A. strigosus* lines served as universal hosts for all thirty *Bradyrhizobium* isolates (hereafter described as the sympatric or universal experiments, respectively). One universal host line originated from a population with high soil nitrogen (CLA; Cla12.04 line) and the other from a population with low soil nitrogen (ANZ; Anz13.04 line). *A. strigosus* lines were descended from wild seeds collected from 2005 to 2012 and generated following published methods in 2014 (Table S3) (Regus *et al.* 2017b). Plant lines from UCR, BMR and ANZ showed 'scaled investment'; the hosts form significantly larger mean nodule size when inoculated with effective vs. ineffective rhizobia, whereas lines from CLA, GRI, and YUC showed 'unscaled investment', wherein hosts do not exhibit significant variation in nodule size dependent on symbiotic quality (Wendlandt *et al.* 2019). The outbred *A. heermannii* seeds came from a source near Hemet, CA (S&S Seeds, Carpinteria, CA).

Inoculation experiments

Seeds were surface sterilised, nick scarified and germinated in sterile ddH_2O. Seedlings were planted into sterilised containers (Stuewe and Sons, Tangent, OR, USA) filled with sterile, nitrogen-free quartzite sand and incubated in a growth chamber for 2 weeks. Seedlings were hardened in a greenhouse under ~ 50% shade (4 days, 1 × daily misting) and fertilised once weekly with sterile nitrogen-free Jensen's solution (Somasegaran & Hoben 1994), beginning with 1 ml per plant and increasing by 2 ml per week until reaching 5 ml per plant, which was used for the duration of the experiment.

The 30 focal *Bradyrhizobium* isolates were grown on agar plates with modified arabinose gluconate medium (MAG) (Sachs *et al.* 2009), and a single colony from each isolate was spread onto five replicate MAG plates and incubated until lawns formed (29 °C, ~ 8 days). Bacterial cells were washed from plates into liquid MAG to estimate concentrations via optical density (Sachs *et al.* 2010a). Cell suspensions were centrifuged (1000 ***g***, 20 min) to remove media and resuspended in sterile ddH_2O at 10^8 cells ml^{-1}. Plants were inoculated with 5×10^8 rhizobial cells in 5 ml sterile ddH_2O (inoculated plants) or with 5 ml sterile ddH_2O (uninoculated controls).

Axenic seedlings of each plant line were size-matched and randomly assigned to inoculation treatments and greenhouse locations in blocks. There were 36 inoculation treatments (6 population sources of isolates × 6 inoculation treatments per site (i.e. average of 5 rhizobial isolates per site + 1 uninoculated control)) × 5 host lines (2 sympatric + 2 universal + *A. heermannii*) × 5 replicates per treatment, except for lines Anz13.04, Anz10.01, Gri01.13, which each had 4 replicates due to poor seedling germination = 852 plants (Fig. S1). Plants were inoculated on 13 March 2015 and harvested 8 weeks later. During harvest, plants were removed from the pots and sand was separated from the roots by washing with tap water (13 May – 26 May 2015). Nodules were separated from roots, counted and photographed. Roots, shoots and nodules were separated and oven dried (60 °C, > 4 days) prior to weighing (Table S4).

Data analysis

Data were $\log_{10}$-transformed as needed to improve normality and were analysed using general linear mixed models (GLMMs) in JMP Pro 13.0 (Fit Model platform, Standard Least Squares, REML method). Means presented in the text are raw means (back-transformed if necessary). All effects were coded as fixed except block, which was treated as a random effect. Nodulation capacity of isolates was assessed by the presence or absence of nodules on tested hosts. Symbiotic effectiveness was quantified as 'plant relative growth', i.e. total plant dry mass of a focal inoculated plant divided by total plant dry mass of its control plant. With no extrinsic source of nitrogen, isotopic analysis has indicated that plant relative growth is an excellent proxy for nitrogen fixation (Regus *et al.* 2014), although plant growth in these conditions is not significantly correlated with overall seed production (Sachs *et al.* 2010a).

We quantified fitness proxies of rhizobia using local genotype frequencies from field samples as well as nodule biomass. The former examines fitness of nodule-forming rhizobia in natural habitats. The latter examines the ability of a rhizobia strain to achieve fitness on specific plant genotypes. Nodule mass is often positively correlated with rhizobial population sizes in nodules of *A. strigosus* (Sachs *et al.* 2010a), *Medicago truncatula* (Heath & Tiffin 2007, 2009), *Glycine max* (Kiers *et al.* 2003), *Lotus japonicus* (Quides *et al.* 2017), and *Lupinus arboreus* (Simms *et al.* 2006). Total nodule mass estimates *in planta* rhizobial fitness at the whole-plant level, and thus accounts for rhizobia that form few but very large nodules or many nodules that are very small. On the other hand, mean nodule mass estimates rhizobial fitness per individual infection. Mean nodule mass factors out nodule number and thus avoids spurious correlations between plant size and rhizobial fitness (since nodule number is correlated with plant biomass)

(Kiers *et al*. 2003; Sachs *et al*. 2010a). A meta-analysis showed that total nodule mass measures are positively correlated with symbiotic effectiveness in clonal inoculation experiments (Friesen 2012), likely because fitness feedbacks are strong when hosts encounter symbiont clones (Kiers *et al*. 2013). However, the majority of these data came from non-native pairings of hosts and rhizobia – excluding the possibility of coevolution (Friesen 2012) – so one focus here is to expand these datasets to native interactions. We investigated evidence of selection on symbiotic effectiveness by testing for correlations between effectiveness on sympatric hosts and local genotype frequency for each genomic region.

For the sympatric experiment, we tested for significant effects of rhizobia isolate, host line (both nested within population source), population source, and block. For the universal experiment, we tested for significant effects of host line, rhizobial isolate, rhizobial isolate × host line and block. Significant effects were explored using Tukey's HSD and linear contrasts of least squares means. To examine the role of soil fertility in shaping rhizobial effectiveness, we tested whether the growth effects of rhizobia (i.e. relative plant growth) differed between high nitrogen population sources (CLA, GRI, UCR) and low nitrogen population sources (ANZ, BMR, YUC) using a post hoc contrast test. Effects of soil nitrogen concentrations on effectiveness were also examined using linear regressions with mineral nitrogen concentration and total nitrogen concentration from published soil data (Regus *et al*. 2017b). Researchers previously defined rhizobia as ineffective when the inoculated hosts grow < 40% compared to hosts inoculated with a highly effective reference genotype (Burdon *et al*. 1999). We used a more conservative cutoff of < 40% symbiotic effectiveness compared to the mean of all isolates within the sympatric dataset. Isotopic analysis found that strains below this cutoff fixed negligible levels of nitrogen for the host (Regus *et al*. 2014).

RESULTS

Nodulation capacity

Twenty-two *Bradyrhizobium* isolates formed nodules on all inoculated plants. Four additional isolates formed nodules on most inoculated plants: isolates 134, 135 and 158 nodulated all but one plant replicate each and isolate 149 had inconsistent nodulation on several *A. strigosus* host lines. Unexpectedly, four isolates failed to nodulate any hosts (133, 140, 148, 161). Isolates 133 and 148 were originally cultured from the *A. strigosus* root surface. Isolates 140 and 161 were originally cultured from nodules, but might have coinfected the original host with a nodulating strain, as has been reported (Gano-Cohen *et al*. 2016). Inoculated plants that did not form nodules were removed from remaining analyses, which focus on the 26 nodule-forming isolates. None of the uninoculated control plants formed nodules. Seven plants died during the experiment, including three control plants. Since control plants were used to calculate relative growth of size-matched inoculated plants, we used data from uninoculated plants in neighbouring blocks to represent the control plants that died (Table 1).

Symbiotic effectiveness; relative growth of inoculated vs. control hosts

Relative growth values were $\log_{10}$ transformed to normalise analyses of symbiotic effectiveness, but raw values are discussed here. In the sympatric experiment, the relative growth effects caused by the nodule-forming rhizobial isolates had a mean of 7.50*x* (i.e. fold increase in biomass compared to uninoculated controls, 95% Confidence Intervals, CI = 6.73–8.36*x*). Only isolate 155 was categorised as ineffective (1.59*x*, CI = 1.15–2.21*x*; YUC); the next-closest isolate to the cutoff was 156 (3.05*x*, CI 2.25–4.15*x*; YUC, Fig. 1a, Fig. S2). In the universal experiment, isolates 155 and 156 were also the least effective, in addition to 149 (from UCR; Fig. 1b). Isolate effectiveness was consistent among universal host lines (i.e. no host × isolate interaction effect; $F_{50,271.8} = 0.6517$ $P = 0.9655$) inconsistent with partner-maladaptation, which predicts conditional effectiveness (Table 1).

We found no evidence for the hypothesis that increased nitrogen concentration in soils favoured the evolution of ineffective rhizobia. A post hoc contrast within the population term of the sympatric host GLMM did not find significant differences in plant relative growth between high and low-nitrogen populations ($F_{1,212.2} = 2.8756$, $P = 0.0914$). The population mean effectiveness of isolates on sympatric hosts was also not predicted by total soil nitrogen ($F_{1,4} = 0.1300$, $P = 0.7367$) or mineral soil N ($F_{1,4} = 0.1072$, $P = 0.7598$). Within the universal dataset, we actually found that isolates from high-nitrogen sites were slightly more effective when we performed a linear contrast test between the 12 isolates from low-nitrogen field sites and the 14 isolates from high-nitrogen field sites ($F_{1,271.9} = 5.9586$, $P = 0.0153$; Table S4).

Total nodule mass; *in planta* rhizobial fitness at the whole-plant level

In the sympatric experiment, total nodule mass per plant had an average value of 7.97 mg (95% CI = 7.44–8.50 mg). Isolate 156 had by far the greatest total nodule mass of all isolates (15.44 mg, CI = 11.99–18.89 mg; Fig. 2a) and 149 had the lowest total nodule mass (3.49 mg, CI = 0.66–6.32 mg). In the universal experiment, isolate 142 (CLA) had the greatest total nodule mass (10.49 mg, CI = 7.50–13.47 mg) and isolate 145 (CLA) had the lowest (5.81 mg, CI = 4.45–7.18 mg; Fig. 2b). Total nodule biomass of isolates was consistent among universal hosts (i.e. no host-isolate interaction effect; $F_{50,271.3} = 0.5753$ $P = 0.9900$), inconsistent with the partner-maladaptation model (Table 1).

Mean nodule mass; rhizobial fitness per individual infection

In the sympatric experiment, mean nodule mass had an average value of 0.17 mg (95% CI = 0.16–0.18 mg). The largest nodules were formed by isolates 134 (BMR; 0.35 mg, CI = 0.24–0.51 mg), 136 (BMR; 0.33 mg, CI = 0.26–0.43 mg), 142 (CLA; 0.26 mg, CI = 0.20–0.35 mg), and 156 (YUC; 0.27 mg, CI = 0.20–0.36 mg) and the smallest nodules formed by isolates 135 (BMR; 0.17 mg, CI = 0.14–0.20 mg), 145 (CLA; 0.09 mg, CI = 0.07–0.11 mg), and 155 (YUC; 0.12 mg,

Table 1 Variation in symbiotic effectiveness and fitness of *Bradyrhizobium* isolates

Source of variation*	Symbiotic effectiveness = $\log_{10}$(Plant relative growth) Adj. R^2 = 0.29, n = 248			Total nodule mass, mg (not transformed) Adj. R^2 = 0.41, n = 245			Mean nodule mass = $\log_{10}$(Mean nodule mass, mg) Adj. R^2 = 0.43, n = 245		
Sympatric hosts†	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Population	5, 212.7	15.4343	**< 0.0001**	5, 209.2	13.1051	**< 0.0001**	5, 208.9	19.3972	**< 0.0001**
Isolate (Population)	20, 212.2	1.8314	**0.0191**	20, 209	3.7724	**< 0.0001**	20, 208.2	4.9163	**< 0.0001**
Host line (Population)	6, 212.8	1.8144	0.0975	6, 209.2	4.9955	**< 0.0001**	6, 209.3	3.4385	**0.0029**
Block, *random*			0.4814			0.2074			0.8131
	Adj. R^2 = 0.41, n = 354			Adj. R^2 = 0.13, n = 353			Adj. R^2 = 0.40, n = 353		
Universal hosts‡	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Host line	2, 274.1	47.1345	**< 0.0001**	2, 273.6	9.9277	**< 0.0001**	2, 273.5	2.7852	0.0635
Isolate	25, 271.8	5.6647	**< 0.0001**	25, 271.4	2.3727	**0.0004**	25, 270.7	7.8378	**< 0.0001**
Host line × Isolate	50, 271.8	0.6517	0.9655	50, 271.3	0.5753	0.9900	50, 270.7	1.9554	**0.0004**
Block, *random*			0.2429			0.3413			0.3090

*We excluded control plants and those that were inoculated but formed no nodules.
†We replaced dead plant #111 (the Anz10.01 control for block 4) with Biomass data from plant #106 (block 3). We replaced dead plant #222 (the Gri01.13 control for block 4) with Biomass data from plant #216 (block 3).
‡We replaced dead plant #669 (the Anz13.04 control for CLA strains in block 4) with the mean biomass of plants #651, #660, #663, #680, #684 (i.e. the ANZ13.04 controls for the other population sources in block 4).
Bold values indicates significant effects in the model.

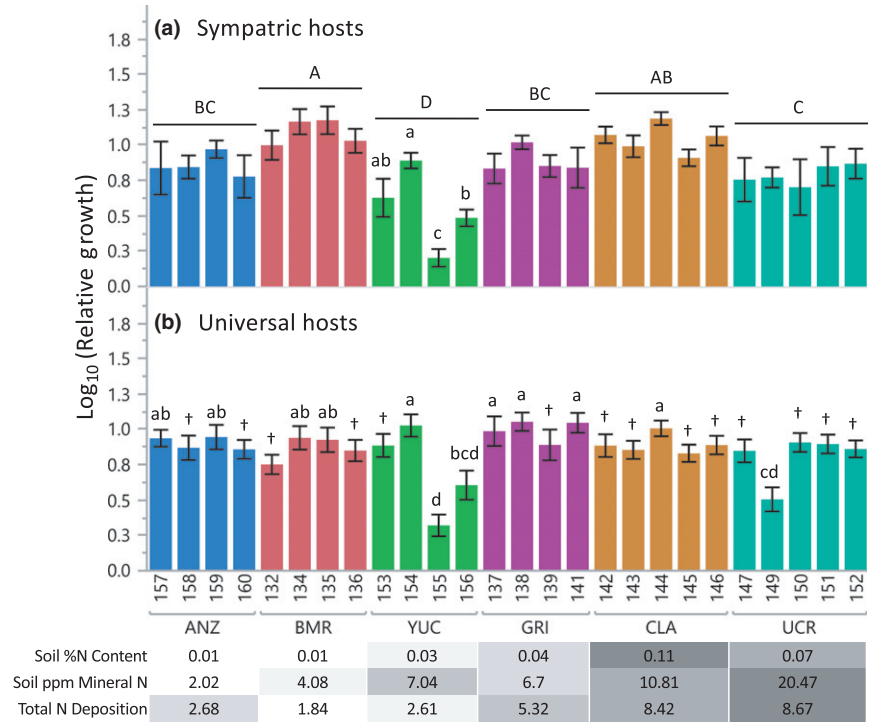

Figure 1 *Bradyrhizobium* effectiveness on universal and sympatric hosts. Variation in effectiveness of *Bradyrhizobium* isolates during inoculations onto (a) sympatric *Acmispon strigosus* hosts (2 lines pooled) and (b) universal hosts (2 *A. strigosus* lines and *A. heermannii*, pooled). Isolates are arranged numerically within their source populations. (a) Different capital letters denote significant differences among population sources by Tukey's HSD. Different lowercase letters denote significant differences among isolates within a population source by contrast analysis. (b) Different lowercase letters denote significant differences among isolates by Tukey's HSD. Daggers are equivalent to 'abc', which we excluded for clarity. Values beneath the main figure indicate soil nitrogen and rates of nitrogen deposition (kg ha^{-1} yr^{-1}) at each population source, with shading proportional to nitrogen concentrations (Regus *et al.* 2017b). Error bars represent ± 1 SE.

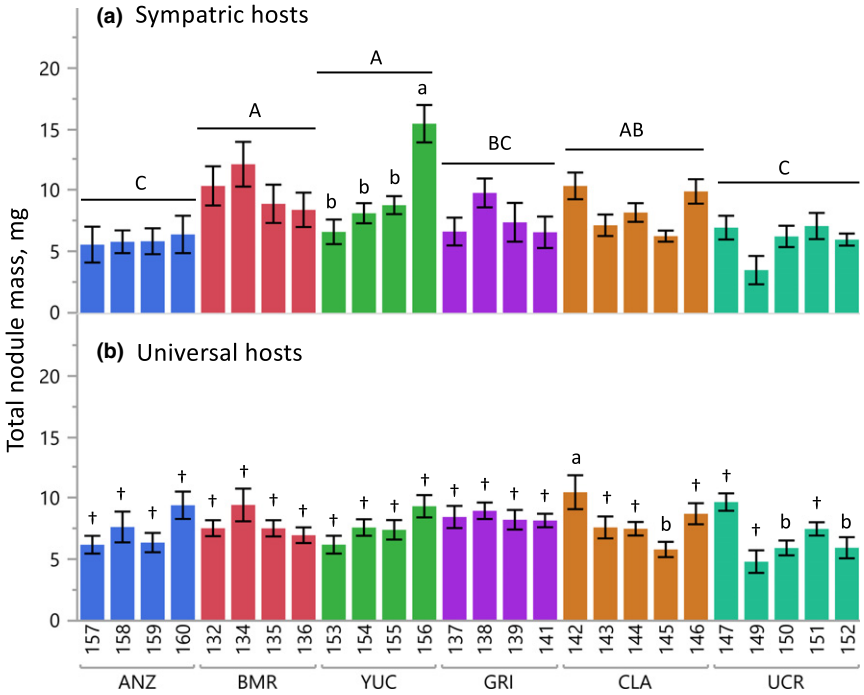

Figure 2 *Bradyrhizobium* total nodule mass on sympatric and universal hosts. Variation in total nodule mass of *Bradyrhizobium* isolates during inoculations onto (a) sympatric *Acmispon strigosus* hosts (2 lines pooled) and (b) universal hosts (2 *A. strigosus* lines and *A. heermannii*, pooled). Isolates are arranged numerically within their source populations. (a) Different capital letters denote significant differences among population sources by Tukey's HSD. Different lowercase letters denote significant differences among isolates within a population source by contrast analysis. (b) Different lowercase letters denote significant differences among all isolates by Tukey's HSD. Daggers are equivalent to 'ab', which we excluded for clarity. Error bars represent ± 1 SE.

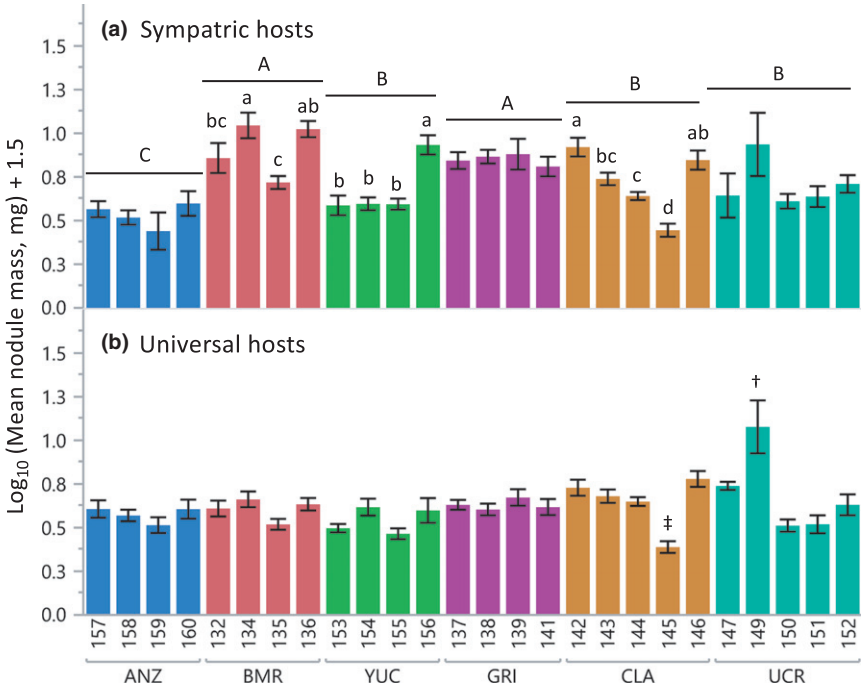

Figure 3 *Bradyrhizobium* mean nodule mass on sympatric and universal hosts. Variation in mean nodule mass of *Bradyrhizobium* isolates during inoculations onto (a) sympatric *Acmispon strigosus* hosts (2 lines pooled) and (b) universal hosts (2 *A. strigosus* lines and *A. heermannii*, pooled). Isolates are arranged numerically within their source populations. (a) Different capital letters denote significant differences among population sources by Tukey's HSD. Different lowercase letters denote significant differences among isolates within a population source by contrast analysis. (b) Isolates marked with a single or double dagger formed the largest or smallest nodules, respectively, on all three universal hosts by Tukey's HSD. The remaining isolates (unmarked) formed intermediate-sized nodules and exhibited host-dependent differences in nodule size (Isolate × Host line interaction). Error bars represent ± 1 SE.

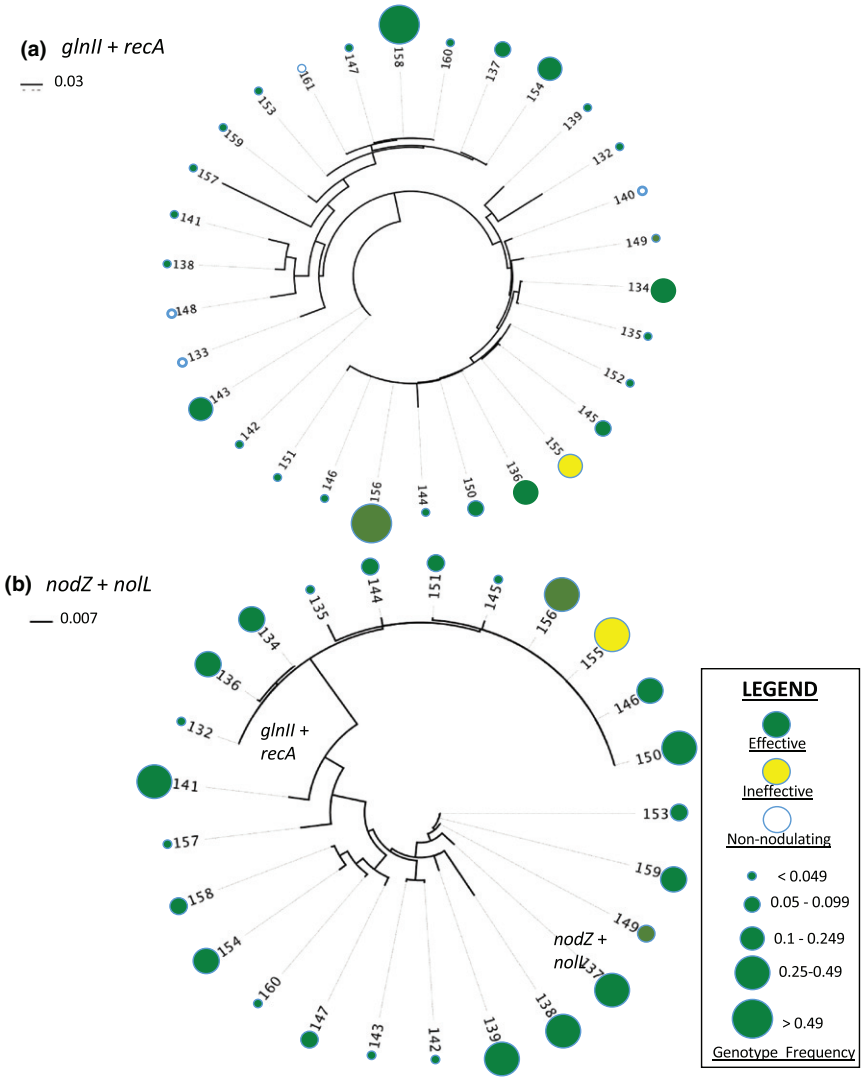

Figure 4 Phylogenetic reconstruction of isolates. Evolutionary trees were reconstructed using CHR loci (a) and SI loci (b) with PhyML 3.0 utilising a BioNJ starting tree and subtree pruning and regrafting. Circles next to isolates are scaled in size to indicate genotypic abundance and are coloured depending on whether the isolate was effective, ineffective, or non-nodulating. Scale bars indicate substitutions per site.

CI = 0.11–0.15 mg; Fig. 3a). In the universal experiment, all host lines formed the largest nodules with isolate 149 (UCR; 0.38 mg, CI = 0.17–0.82 mg) and the smallest nodules with isolate 145 (CLA; 0.08 mg, CI = 0.07–0.09 mg; Fig. 3b). Mean nodule biomass of isolates varied among universal hosts (significant host–isolate interaction effect; $F_{50,270.7} = 1.9554$, $P = 0.0004$), hence that some rhizobia genotypes formed relatively large nodules on host genotypes but small nodules on others, consistent with partner-maladaptation.

Association between *Bradyrhizobium* effectiveness and fitness

First, we tested whether individual *Bradyrhizobium* genotypes fulfilled the operational definition of a cheater proposed by Jones and colleagues (Jones *et al.* 2015), by providing less benefit to partners compared to the sympatric population mean but exhibiting superior fitness upon those partners, in terms of total nodule mass and mean nodule mass. Two *Bradyrhizobium* isolates fit these criteria, including isolate 160 (ANZ) and 156 (YUC; Figs 1a, 2a and 3a). However, only isolate 156 also had a local genotype frequency above the population mean for both CHR and SI sequences (Table S2, Fig. 4), consistent in the strictest sense with the Jones and colleagues definition of cheating (Jones *et al.* 2015).

We also analysed statistical associations between *Bradyrhizobium* effectiveness and fitness proxies to test the partner-maladaptation and interspecific conflict hypotheses (Sachs *et al.* 2004; Jones *et al.* 2015; Sachs 2015). We analysed linear regressions between symbiotic effectiveness on sympatric hosts and genotypic proxies of *Bradyrhizobium* fitness and found that symbiotic effectiveness negatively predicted the local genotype frequency for both the CHR and SI loci (CHR: $F_{1,24} = 4.468$, $R^2 = 0.1570$, $P = 0.0451$; SI: $F_{1,23} = 4.996$, $R^2 = 0.1784$, $P = 0.0352$; Fig. 5). These data are consistent with directional selection favouring rhizobia that provide less

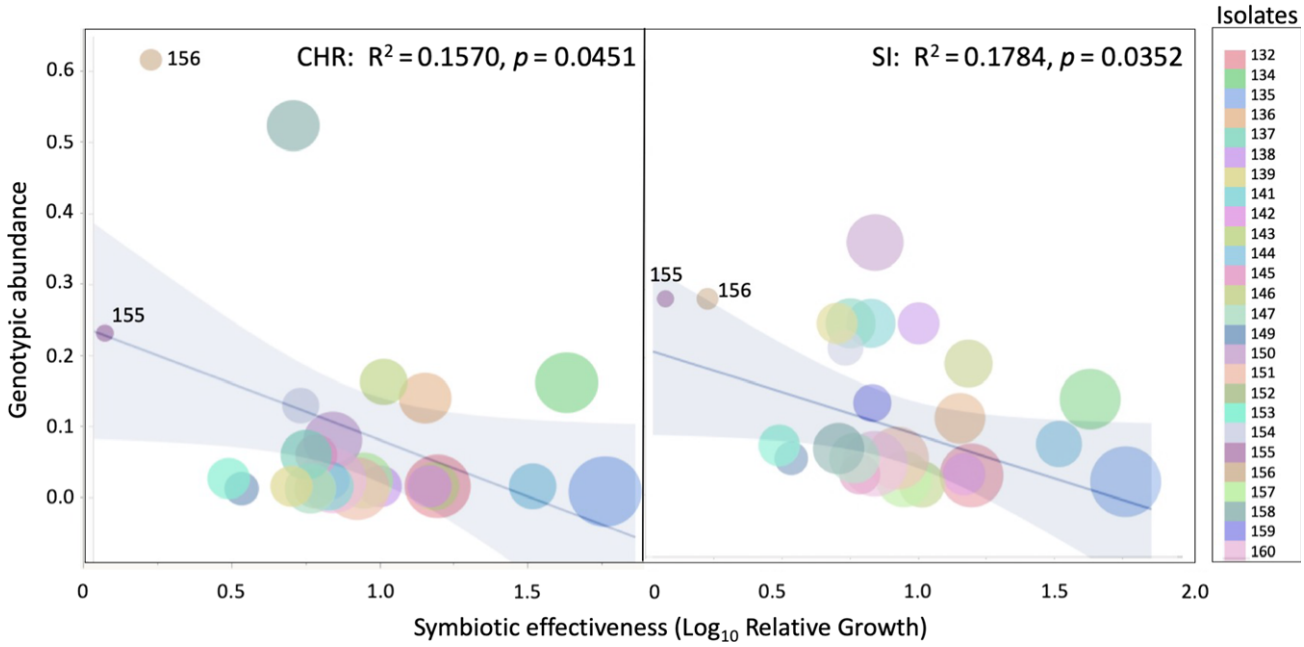

Figure 5 Correlation between effectiveness and local genotype frequency. Symbiotic effectiveness is negatively correlated with genotype frequency, consistent with directional selection favouring less-beneficial rhizobial genotypes. The left graph shows measures of genotype frequency using the chromosomal loci (CHR) on the *Y* axis and the right graph shows measures of genotype frequency using the symbiosis loci, in each case frequencies of each genotype are quantified separately for each population. Symbiotic effectiveness on the *X*-axis is measured via the growth response of sympatric host lines relative to uninoculated control hosts. Each point is coloured by strain and the diameter of each point displays the standard error of HGR for that isolate. Isolates #155 and 156 are highlighted because they were the least beneficial strains across the metapopulation.

benefit to local hosts, and support the interspecific conflict model.

Finally, we tested for statistical associations between *Bradyrhizobium* effectiveness and *in planta* fitness. However, there was no statistical association between effectiveness and nodule biomass for the sympatric dataset (Total nodule mass: $F_{1,24} = 0.1089$, $R^2 = 0.0045$, $P = 0.7442$, Log_{10} Mean nodule mass: $F_{1,24} = 1.643$, $R^2 = 0.0641$, $P = 0.2121$) or the universal dataset (Total nodule mass: $F_{1,24} = 0.8968$, $R^2 = 0.0360$, $P = 0.3531$, Log_{10} Mean nodule mass: $F_{1,24} = 0.6175$, $R^2 = 0.0251$, $P = 0.4397$). In testing for consistency among fitness proxies, CHR but not SI frequency was predicted by total nodule mass (CHR, $F_{1,24} = 5.03$, $R^2 = 0.180$, $P = 0.035$; SI, $F_{1,24} = 1.61$, $R^2 = 0.065$, $P = 0.218$). There was not a significant correlation between the CHR and SI frequencies ($F_{1,24} = 1.502$, $R^2 = 0.061$, $P = 0.232$), consistent with previous evidence of horizontal gene transfer of the SI (Hollowell *et al*. 2016b).

DISCUSSION

To our knowledge, this study is the first to simultaneously test for cheating and the selective conditions that favour cheating in a metapopulation of mutualists. We uncovered *Bradyrhizobium* genotypes bearing traits that are consistent with models of cheating in mutualism. Isolates 156 and 160 both provided less net benefit to hosts – when compared to other rhizobia in their local population – while exhibiting superior *in planta* fitness upon those hosts (i.e. consistent with cheaters; Jones *et al*. 2015). We also uncovered evidence consistent with directional selection favouring cheating; the net growth benefit that *Bradyrhizobium* strains provided to sympatric hosts was negatively associated with their genotype frequency in those populations. Similarly, a selection gradient that favours cheating was also uncovered in *Ensifer medicae* (formerly *Sinorhizobium*), wherein the rhizobia strains that provide less benefit to hosts consistently exhibit superior fitness on those plants (Porter & Simms 2014). Unlike our study, Porter & Simms (2014) found this correlation for nodule size, but their study provided a better estimator of selection on this parameter as mean values were analysed across 18 different host lineages. These data build on decades of work uncovering mechanisms by which rhizobia can exploit their interactions with legumes. For instance, the *Bradyrhizobium elkanii* strain USDA61 produces rhizobitoxine, an ethylene inhibitor that blocks the host plant's capacity to regulate nodulation, and meanwhile USDA61 fixes little nitrogen, forms many nodules, and competes successfully against other strains for nodulation (Yuhashi *et al*. 2000; Ma *et al*. 2002). In *Sinorhizobium meliloti*, some strains bear the *hrrP* locus, which is necessary and sufficient to inhibit nitrogen fixation while causing the rhizobia to induce 5–10 times more nodules and to hyper-proliferate within nodule tissue (Price *et al*. 2015). In both cases host defense has evolved suggesting an arms race between symbionts and hosts (Sachs *et al*. 2018). Soybean has evolved a resistance allele that terminates rhizobitoxine producing *Bradyrhizobium* (Yasuda *et al*. 2016) and the effects of the *Sinorhizobium hrrP* locus are also blocked by some host lines (Price *et al*. 2015).

Fitness conflict occurs in diverse mutualisms, including intimate host-microbe associations like was studied here.

Dinoflagellate algae (*Symbiodinium*) inhabit marine invertebrate hosts and provide them with photosynthates in exchange for nitrogen, but many *Symbiodinium* strains come to dominate hosts while providing the host with little or no fitness benefits (Sachs & Wilcox 2006; Stat *et al*. 2008; Stat & Gates 2011). Similarly, some plant hosts have evolved to exploit ectomycorrhizal root associates by transitioning to a non-photosynthetic lifestyle that requires using root fungi as a conduit of resources taken from other plant hosts (Bidartondo & Bruns 2001). Mutualisms that occur between non-microbial partners are also rife with conflict. In pollination mutualisms, for instance, both plants and pollinators have evolved to exploit the other, either by producing nectarless flowers (Smithson & Gigord 2003) or by stealing nectar without pollinating (Irwin *et al*. 2010). In cleaner–client mutualisms, wherein members of one species remove ectoparasites from a client species in exchange for food or protection, the cleaner can opportunistically parasitise the client by opening wounds or feeding on blood (Bshary 2002; Douglas 2008; Sachs 2015). In many cases, cheaters and mutualists might coexist stably because of tradeoffs in key fitness parameters such as competitive ability, colonisation rate and fecundity (Jones *et al*. 2009). Only rarely has evidence suggested that exploiters cause a mutualism to collapse or to transition to parasitism (Sachs & Simms 2006).

We also examined evidence for partner-maladaptation, the hypothesis that ineffective rhizobia are specific to (and effective on) a different host genotype or species, in which case neither partner gains much benefit from the mismatched association (Sachs & Simms 2008; Friesen 2012). However, the evidence we uncovered was largely inconsistent with maladaptation. Firstly, the universal experiment largely mirrored the sympatric experiment in terms of relative growth effects of the different rhizobia isolates on the hosts. Secondly, the two least effective isolates of rhizobia that we uncovered in all populations (155, 156) had average to above-average fitness by all measures. Finally, we found no evidence for G × G interactions for symbiotic effectiveness or total nodule biomass, our main proxies of rhizobial fitness effects upon the host and rhizobial fitness. We did, however, find evidence of a significant G × G interaction for mean nodule biomass in the universal experiment analysis, suggesting that specificity interactions can sometimes influence host investment into individual infections. These data suggest that specificity might not be strong in *Acmispon–Bradyrhizobium* interactions, at least among the host and rhizobia genotypes that we tested.

In conclusion, we found that variation in symbiotic effectiveness of rhizobia is most consistent with interspecific conflict, but the specific conditions promoting ineffective rhizobia require further investigation. One prediction is that legume defenses degrade in nitrogen rich soils where hosts gain little benefit from nodulation (Regus *et al*. 2014, 2017b) thus leading to the evolution of ineffective rhizobia. However, we found no evidence to support this hypothesis. In parallel, several studies found that legumes did not downregulate defenses (i.e. phenotypic plasticity) or evolve reduced defenses when exposed to nitrogen fertilisation (Kiers *et al*. 2006; Regus *et al*. 2014; Wendlandt *et al*. 2019). A 22-year experimental fertilisation regime resulted in rhizobia with reduced effectiveness compared to control plots (Weese *et al*. 2015), but it is unclear whether these changes were driven by host plasticity, evolution, or something else, such as a change in the plant community. The nitrogen fertilisation used by Weese and colleagues was ~10x the deposition rates at our most polluted sites, and *Acmispon* hosts are sickly and fail to nodulate at these concentrations (Regus *et al*. 2017b). A broader prediction is that host defense traits are costly, and evolve dependent on the net benefits of symbiosis (Foster & Kokko 2006; Steidinger & Bever 2014, 2016). We uncovered two low-quality rhizobia in a population where *Acmison* lines exhibit an unscaled strategy for investment into rhizobia (Wendlandt *et al*. 2019), suggesting that some host variants might provide a refuge for low quality mutualists. Legume hosts can also vary in their diversity of associated rhizobia (Ehinger *et al*. 2014) and generalism can be favoured over partner specialisation when diverse mutualist partners are available (Batstone *et al*. 2018). Thus, just as variation in legume defense traits might lead to a coevolutionary response in rhizobia, evolution of generalism or specialisation, might drive variation in rhizobial effectiveness (Ehinger *et al*. 2014). Future research is needed to examine the spatiotemporal and genomic drivers of rhizobial effectiveness and host defense to reveal whether these traits are shaped by ongoing coevolutionary conflict.

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AUTHORSHIP

KGC designed the study, performed the experiment, analysed the data, and wrote the manuscript. CEW designed the study, performed the experiment, contributed substantially to revisions. PJS, MAB, KWQ, AZ, and ESA helped perform the experiment and collect data. JLS designed the study and wrote the manuscript. KGC, CEW, and JLS contributed substantially to revisions.

COMPETING INTERESTS

The authors have no competing interests.

DATA ACCESSIBILITY STATEMENT

The data supporting the results in this manuscript will available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.cr65269.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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