The genetic basis of cooperation and conflict in natural populations of a model symbiont

Rebecca T. Batstone[*,1], **Liana T. Burghardt**[†] **and Katy D. Heath**[*,‡]

[*]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, IL 61801 USA, [†]Department of Plant Science, The Pennsylvania State University, 103 Tyson Building, University Park, PA, 16802 USA, [‡]Department of Plant Biology, University of Illinois at Urbana-Champaign, 286 Morrill Hall, 505 South Goodwin Avenue, Urbana, IL 61801 USA

ABSTRACT Although mutualisms are defined as net beneficial interactions among species, whether fitness conflict or alignment drive the evolution of these interactions is unclear. Examining the relationships between host and symbiont fitness proxies at both the organismal and genomic levels can provide new insights. Here, we utilized data from several genome-wide association studies (GWAS) that involved 191 strains of the N-fixing rhizobium symbiont, *Ensifer meliloti*, collected from natural populations being paired in single or mixed inoculation with two genotypes of the host *Medicago truncatula* to determine how different proxies of microbial fitness were related to one another, and examine signatures of fitness conflict and alignment between host and symbiont at both the whole-organism and genomic levels. We found little evidence for fitness conflict; instead, loci tended to have concordant effects on both host and symbiont fitness and showed heightened nucleotide diversity and signatures of balancing selection compared to the rest of the genome. We additionally found that single versus competitive measures of rhizobium fitness are distinct, and that the latter should be used given that they better reflect the ecological conditions rhizobia experience in nature. Our results suggest that although conflict appears to be largely resolved in natural populations of rhizobia, mutualistic coevolution between legumes and rhizobia can nonetheless maintain genetic diversity, potentially explaining why variation in symbiotic traits persists in nature.

KEYWORDS conflict; cooperation; GWAS; symbiosis; pleiotropy

Introduction

A central goal of genetics research is to understand how genotypes give rise to organismal phenotypes, in other words to develop a reliable map of genotype-to-phenotype relationships (G→P map; Mackay *et al.* 2009; Pigliucci 2010; Lehner 2013) that can be used to pinpoint the genetic basis of important traits such as plant yield in agriculture or disease-related traits in humans. Recent advances in sequencing and microbiome research have revealed the ubiquity of symbioses, intimate interactions among unlike organisms (*sensu* de Bary, 1878), and thus that many of these 'macrobial' host phenotypes (disease resistance, yield, etc.) are actually symbiotic extended phenotypes, influenced at least in part by loci present in interacting microbial symbionts (Heath 2010; Friesen *et al.* 2011; Ferrari and Vavre 2011; Gould *et al.* 2018; Lynch and Hsiao 2019; O'Brien *et al.* 2021). While symbiosis has most often been studied from the perspective of ecological outcomes (Bronstein 1994; Kiers *et al.* 2010; Hoeksema 2010; Chamberlain *et al.* 2014; Simonsen and Stinchcombe 2014a; Hoeksema and Bruna 2015) or even metabolic complementarity (Wu *et al.* 2006; McCutcheon and Moran 2007; McCutcheon and Von Dohlen 2011; Sabree *et al.* 2012), there is increasing recognition of intraspecific, quantitative genetic variation in symbiotic extended phenotypes, implying complex and environmentally-dependent genetic effects (Heath and Stinchcombe 2014; Ostrowski *et al.* 2015; Stoy *et al.* 2020; O'Brien *et al.* 2021). Thus predicting how symbiotic extended phenotypes evolve in nature requires an understanding of how these complex traits arise from the intimate interaction of multiple genomes.

Studies of the G→P map in single organisms have highlighted the importance of pleiotropy, i.e., genes or mutations that influence more than one trait, with important consequences

doi: https://doi.org/10.1101/2021.07.19.452989
Manuscript compiled: Tuesday 20th July, 2021, using the Overleaf template available at https://www.overleaf.com/gallery/tagged/gsa
[1]Corresponding author: Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, IL 61801 USA.
E-mail: rtbatstone@gmail.com

for development, aging, and disease (Stearns 2010; Wagner and Zhang 2011). In single organisms, pleiotropy can constrain adaptation when it underlies trade-offs among fitness-related traits (Stearns 1989; Barton 1990; Roff *et al.* 2000; Otto 2004). In the G→P map of symbiotic extended phenotypes, pleiotropic loci can also underlie traits of interacting individuals, which we henceforth refer to as symbiotic pleiotropy. In symbiotic mutualisms, wherein partners trade fitness benefits (Bronstein 2015), symbiotic pleiotropy can couple the traits that benefit the individual (private traits) with traits that benefit the partner (cooperative traits; Foster *et al.* 2004; Mitri and Foster 2016; Dos Santos *et al.* 2018; Queller 2019), promoting cooperation. The directionality of symbiotic pleiotropy is critical because it determines the degree to which partners' fitnesses are aligned (i.e., same-sign or concordant effects) or in conflict (i.e., opposite-sign or discordant effects), and thus, defines the mutational landscape of mutualism evolution in nature. Although mutualisms are defined as net beneficial interactions among species, whether fitness conflict or alignment drive the evolution of these interactions is hotly debated (Jones *et al.* 2015; Frederickson 2017). If symbiotic pleiotropy is extensive, and its effects on fitness-related traits in the interacting partners tend to be discordant (i.e., increases in one partner's fitness come at the expense of the other partner), then conflict should underlie the evolution of mutualism, allowing for the possibility of cheating individuals that are competitively superior, as mutualism theory predicts (Bull and Rice 1991; West *et al.* 2002a,b; Foster and Kokko 2006; Dos Santos *et al.* 2018; Queller 2019). On the other hand, if pleiotropic effects are overwhelmingly concordant (i.e., effects on host and symbiont fitnesses are in the same direction), fitness alignment rather than conflict should be the null hypothesis in mutualism, and models relying on cheating genotypes as the main driver of mutualism evolution may not be suitable.

Such host-symbiont conflict may be measured at the whole organism level (i.e., do low-quality individuals derive higher fitness from symbiosis?) or at the genomic level (i.e., do alleles that increase one partner's fitness decrease the other partner's fitness?). Results at these two levels can differ; for instance, fitness alignment between the sexes prevails at the phenotypic level, but sexually-antagonistic loci nonetheless exist and can have important consequences on the coevolution of sexual traits (Innocenti and Morrow 2010). Genome-wide association studies (i.e., GWAS) have the potential to reveal the genes, as well as specific variants, that underlie phenotypic variation within key mutualism traits in natural populations (Klinger *et al.* 2016; Burghardt *et al.* 2018; Epstein *et al.* 2018; Batstone *et al.* 2020a). Because they provide an estimate of both the strength and direction of effects of particular alleles on the phenotype, GWAS of natural populations are useful for addressing broad questions about the effects of standing genetic variation in nature, and can facilitate the study of how historical selection has acted on those traits in the past (Mackay and Huang 2018; O'Brien *et al.* 2021). While organismal-level genetic correlations reflect the action of all contributing alleles as they are packaged into genotypes, GWAS has the power to reveal individual allelic effects "unpackaged" from those multilocus genotypes. Thus GWAS can reveal variants underlying fundamental sources of conflict at the genomic level even if fitness alignment is realized at the whole organism level due to linkage disequilibrium among genotypes arising from various processes including physical linkage, historical epistatic selection, or even neutral drift/population structure (Conner *et al.* 2004; Lynch and Walsh 1998).

Over the past ∼25 years, legume-rhizobium symbioses have been developed as models for understanding mutualism evolution (Parker 1995; Denison 2000; Kiers *et al.* 2003; Simms *et al.* 2006; Heath and Tiffin 2009). This interaction is one of the most ecologically and economically important symbioses, contributing upwards of 20 million tonnes of nitrogen (N) to the global N-cycle, and saving billions of dollars that would have otherwise been spent on synthetic N fertilizer production (Foyer *et al.* 2016). Legumes house rhizobia within specialized root nodules and supply them with photosynthate, while rhizobia within the nodules convert atmospheric N to a plant-usable form, resulting in a beneficial exchange of resources. Key traits of this symbiosis, such as plant biomass and nodule number, are symbiotic extended phenotypes known to be influenced by variants in the genomes of both host and symbionts, as well as the epistatic interactions between them (Genotype-by-Genotype or G x G interactions; e.g., Parker 1995; Burdon and Thrall 1999; Heath 2010; Heath *et al.* 2012; Burghardt *et al.* 2017; Epstein *et al.* 2018; Batstone *et al.* bioRiv).

Addressing patterns of conflict versus alignment over symbiotic traits requires robust proxies of fitness. Seed number, or close correlates such as plant biomass and leaf chlorophyll A, are well-established proxies for reproductive fitness in annual plants (Friesen *et al.* 2014; Simonsen and Stinchcombe 2014b; Heath and Tiffin 2007). Estimating rhizobium fitness, particularly before the advent of second generation sequencing and improved metagenomic methods, has been an empirical and conceptual challenge, and thus has received much attention in the literature (Denison 2000; Ratcliff *et al.* 2011; Oono *et al.* 2011; Friesen 2012; Kiers *et al.* 2013; Friesen and Heath 2013). Nodule number and nodule size have both been used as fitness proxies because a rhizobium that establishes a nodule in symbiosis can gain a fitness benefit on the order of $10^5 - 10^7$ (Denison and Kiers 2011), larger nodules release more rhizobia (Moawad *et al.* 1984; Heath and Tiffin 2007; Oono *et al.* 2009; Ratcliff *et al.* 2011; Porter and Simms 2014), and intuitively strains that produce more nodules will release more rhizobia (Heath and Tiffin 2009; Heath 2010; Porter and Simms 2014). While single inoculation experiments are required for simultaneously estimating genotypic means for both partners, they cannot address competition among strains in real populations. Until recently, competition could be studied with only a few strains, often using isogenic mutants (e.g., Heath and Tiffin 2009; Friesen 2013; Simonsen and Stinchcombe 2014b). Recent advances merging population genomics and metagenomics allow us to measure a strain's ability to compete for nodulation opportunities and for host investment in nodule growth (Burghardt *et al.* 2018, 2019) and hinted at a surprising lack of correlation between nodule number and competitive fitness, motivating our comprehensive analysis here.

A quantitative comparison of single-strain and multi-strain measures of rhizobium fitness, as well as their correlations with host fitness, is needed to decouple individual strain fitness from host growth and potentially reveal interspecies conflict. Early attempts to address conflict between legumes and rhizobia relied on single-strain inoculation experiments; here, negative correlations between rhizobium partner quality and rhizobium fitness (i.e., nodule number) were interpreted as evidence for conflict between host and symbiont fitnesses (Heath 2010; Simonsen and Stinchcombe 2014b; Porter and Simms 2014). In contrast, mutant and experimental evolution studies have suggested a lack of rhizobial mutations underlying conflict (Friesen 2012; Batstone *et al.* 2020a). However, these experiments have been criticized

for producing spurious positive correlations due to stronger fitness feedback between host plants and rhizobia in single strain inoculation, versus when multiple competing strains are present (Kiers *et al.* 2013), particularly because competition is likely a critical component of fitness in natural and agricultural soils where many rhizobial strains coexist. Thus detecting conflict between hosts and rhizobia might require multi-strain inoculation experiments wherein the benefits to individual strains in a mixed population can be decoupled from overall plant growth.

Here we combine two datasets: first, phenotypic variation and underlying allelic effects from a GWAS of plant and rhizobium fitness proxies measured in single-strain inoculation across 191 strains of *Ensifer meliloti* from natural populations on each of two host genotypes, with a second dataset measuring competitive fitness of 89 of these strains on each of the two host genotypes. We combine these datasets to first ask: What are the relationships among rhizobium fitness components at both the organismal- and genomic-levels? Are these traits genetically distinct and thus likely to evolve independently, or are these linked through pleiotropy? We use this information to address the potential for genomic conflict in this symbiosis, asking: Do segregating mutations tend to have aligned or conflicting effects on host and symbiont fitnesses? Finally we ask whether there is any evidence that historical selection has differentially shaped alleles underlying alignment versus conflict, as we might predict under different models of mutualism evolution.

Materials and Methods

Study system

Full details are provided in Riley *et al.* (2021) and Batstone *et al.* (bioRiv). *Ensifer* (formerly *Sinorhizobium*) *meliloti* is an Alphaproteobacteria that forms symbiosis with various *Medicago* spp., fixing atmospheric N in return for plant photosynthate. All 191 strains used here were collected from the native range of the host *Medicago truncatula*, spanning Spain to France, as detailed in Riley *et al.* (2021). *E. meliloti* has a multipartite genome, including a large (∼3.7 Mbp) chromosome and two symbiosis plasmids (pSymA and pSymB, ∼1.4 Mbp and ∼1.7 Mbp, respectively), the latter known to contain many canonical genes for nodulation and N-fixation (Finan *et al.* 2001; Galibert *et al.* 2001). We used two lines of *M. truncatula* DZA 315.16 (hereafter DZA) and A17 in separate single inoculation experiments, and a mixed inoculation experiment detailed below.

Single inoculation experiments

Full details are provided in Batstone *et al.* (bioRiv). Briefly, we conducted two separate experiments in Sept and Nov 2018, one for each *M. truncatula* line, DZA and A17, respectively. At least three replicate seeds of each plant line were singly inoculated with one of 191 strains of *Ensifer meliloti* isolated from soil spanning *M. trunatula*'s native range (Grillo *et al.* 2016; Riley *et al.* 2021). Seeds were prepared according to published protocols (Barker *et al.* 2006). Briefly, seeds were nick- or sandpaper-scarified, surface sterilized in EtOH for 30s, bleached (6% hypochlorite) for 7 min, rinsed with dH_2O until bleach was completely removed, and imbibed in dH_2O overnight in the dark at 4°C. For planting, we used SC10R Cone-tainers (Stuewe & Sons Inc., Tangent, OR), filling each with autoclaved potting media consisting of one part root wash (1:1:1 mix of field soil:torpedo sand:Turface) and one part Turface MVP calcined clay (Profile Products LLC, Buffalo Grove, IL, USA). A single seed was sown into each pot, and pots were completely randomized with respect to treatment across three greenhouse benches located at the University of Illinois at Urbana-Champaign (UIUC). We cultured rhizobia by loop-inoculating sterile liquid tryptone-yeast (TY) medium (Somasegaran and Hoben 1994) with each strain individually, placing them into a shaking incubator set to 30°C and 200 rpm overnight. We measured the OD_{600} of each culture, and diluted to $\sim 10^6$ cells per ml using sterile TY media to ensure all plants were inoculated with the same initial starting densities. Plants were misted throughout the experiment using upright misting nozzles (1/2" M NPT, Senninger Irrigation Inc., Clermont FL), and harvested six to seven weeks post-inoculation.

Within each experiment, we measured proxies for both rhizobia quality (shoot biomass, leaf chlorophyll A content) and rhizobium fitness (nodule number, per nodule weight). We measured chlorophyll using a SPAD 502 Plus (Spectrum Technologies, Inc. Aurora, IL, USA), recording the mean of three separate measurements on the more recently emerged leaf. Once plants were harvested, all nodules were counted, and ten nodules were haphazardly chosen to be weighed to the nearest 0.001 mg, dividing this number by ten to obtain a per-nodule average for each plant. Shoots were placed in a drying oven set to 60°C and left for three days, being weighed to the nearest 0.01 g. Contamination was minimal in either experiment: we only found 24 nodules on six internal controls (range: 1-9 per plant), and no nodules on external controls.

Mixed inoculation experiment

Briefly, we grew A17 and DZA hosts with a mixed inocula composed of an 89 strain subset of those used in the previous experiment. We inoculated each strain into one of 89 culture tubes filled with 3 ml of TY media (6 g tryptone, 3 g yeast extract, 0.38 g $CaCl_2$ per L) (Somasegaran and Hoben 1994) and grew cultures to stationary phase by placing the tubes in a 120 rpm shaker for 72 hours at 28°C. We combined all cultures, mixed well, and used this mixture to inoculate 3-day old seedlings with $\sim 10^7$ rhizobial cells diluted in 25 ml of N-free fertilizer. Three days prior to inoculation, we filled twelve Cone-tainers with steam-sterilized SUN GRO LP5 Sunshine mix and planted each with four seeds of either A17 or DZA. These seeds had been sterilized in 10% bleach, razor-blade scarified, and cold, dark stratified on petri plates for 2 days at 4°C. After two weeks plants were thinned to ∼3 plants per pot and were provided with nitrogen-free fertilizer (Bucciarelli *et al.* 2006) and sterile H_2O as needed. These plants spent the first month of life in a growth chamber and then were moved to the greenhouse. After twelve weeks we harvested and pooled all nodules from each replicate pot, sterilized them for 30 seconds in 10% bleach, rinsed them thoroughly, and placed the nodules in a 15 ml falcon tube with 3 ml of sterile 0.85% NaCl solution. We homogenized nodules for 1-2 min at the highest speed using a handheld tissue homogenizer (Omni International, Kennesaw, GA). We then enriched for undifferentiated rhizobia using centrifugation: first a 10 min slow spin at 400 x g to remove the heavy plant and bacteroid debri and then subjected the supernatant to a fast spin at 16,000 x g to pellet the remaining undifferentiated rhizobial cells. These pellets were frozen and we used a Plant DNeasy kit to extract DNA from both nodule pellets and the initial inoculum. Next we generated NexteraXT libraries obtained 250 bp paired-end reads on an Illumina NextSeq to ∼120x coverage. After trimming and cleaning, SNP variant frequencies were called by aligning reads to the USDA 1106 reference genome, finally HARP was used to

infer the relative frequency of each strain in each pool (Kessner *et al.* 2013). Sequencing revealed an unintended strain of *Ensifer medicae* in the community. This *E. medicae* strain comprised less than 0.3% of the original mixture and less than .01% of the final nodule community in either host. We omitted this strain from further analysis. Lastly, we calculated overall strain fitness as the fold change in the frequency of a strain (q_x) after selection relative to its mean frequency across the four sequencing replicates of the initial community $log2(q_x selected/q_{\bar{x}} initial)$. This transformation controls for slight differences in initial strain frequency and leads to a normally-distributed trait for downstream analysis. Full details and explanation of the Select and Resequence methodology is provided in Burghardt *et al.* (2018). Raw read data can be found at NCBI BioProject: PRJNA388336.

Phenotypic analyses

As described in Batstone *et al.* (bioRiv), we calculated the estimated marginal means for each trait in each experiment, correcting for the effect of rack, using the emmeans package (v1.4.1 Searle *et al.* 1980), in the R environment (Team 2016). We then conducted linear pairwise regressions (lm option in base R) for each trait against the other within each plant line, as well as the same trait between the two plant lines. We calculated the Pearson correlation coefficients using the "complete observation" option of the cor function in the corrplot package (v0.88, Taiyun and Viliam 2017), and calculated adjusted p-values using the "fdr" (false discovery rate) method of the corr.test function of R's psych package (v1.8.12, Revelle 2018). To visualize the resulting genetic correlations, we used a custom function that utilizes ggplot (v3.3.3, Wickham 2011) to produce the paneled figures.

DNA isolation, whole-genome sequencing, and variant calling

Detailed methods are provided in Riley *et al.* (2021). Briefly, in order to obtain DNA from each of the 191 rhizobia isolates, we grew overnight cultures as described above, and used the Qiagen DNeasy kit (Hilden, Germany) to extract and purify DNA, as per the manufacturer's protocol. We then submitted DNA samples to be sequenced by the DOE Joint Genome Institute (JGI) for sequencing (Berkeley, CA, USA), using the Illumina HiSeq-2500 1TB platform (Illumina, Inc., San Diego, CA, USA) with 100 bp paired-end reads. We recovered only 166 whole-genome sequences that passed quality thresholds, and thus, we regrew 25 of the 191 strains, extracted their DNA using the Zymogen Quick-DNA kit for Fungi or Bacteria (Irvine, CA, USA), and then submitted these samples to be sequenced by the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign (USA) using the Novaseq 6000 platform (Illumina, Inc, San Diego, CA, USA) with 150 bp paired-end reads. We passed the resulting raw reads through a standard bioinformatics pipeline involving read trimming (TrimGalore!: https://github.com/FelixKrueger/TrimGalore; HT-Stream: https://github.com/s4hts/HTStream), alignment to the reference strain *Ensifer meliloti* UDSA 1106 (assembly ID: GCA_002197065.1) using the bwa mem algorithm with default settings (v0.7.17-r1188, Li and Durbin 2009), and variant calling using FreeBayes (v1.3.1-16, Garrison and Marth 2012). We hard-filtered the resulting 491,277 variants using vcftools (v0.1.17, Danecek *et al.* 2011), reducing the set to 36,526 variants with quality scores above 20, minor allele frequencies (MAF) of $\leq 5\%$, minimum mean depth of 20 and a maximum mean depth of 230, successful genotype calls for at least 80% of the strains, and removed any sites that differed between our samples and the reference strain, but were invariant among our samples.

Genome-wide association tests

We conducted linear mixed models (LMMs) implemented in GEMMA (v0.98.1, Zhou and Stephens 2014) to quantify the strength of the association between variants and rhizobium quality and fitness traits. We ran eight separate association analyses in GEMMA, one for each of the four traits from the single-strain inoculation experiment (leaf chlorophyll A content, shoot biomass, nodule number, nodule weight) and host line (DZA, A17; four traits x two hosts = eight runs), while for the subset of 89 strains used in the mixed inoculation experiment, we ran association analyses only on one trait (competitive fitness) for each of the two host lines, for a grand total of 10 association analyses.

Because association analyses rely on variants being statistically distinguishable, we first had to identify variants in strong linkage disequilibrium (LD), picking a representative variant within each LD group using the method employed in Epstein *et al.* (2018). Briefly, we calculated the pairwise LD among all quality-filtered variants, grouping variants in strong LD (95%), and then picking the variant within each LD group exhibiting the highest minor allele frequency, and then the least missing data (if ties were present). This LD grouping analysis resulted in ~8,650 representative variants that went on to be tested in association analyses: 757 on the chromosome, 3,277 on the pSymA, and 4,612 on the pSymB. We used the -lmm 4 option in GEMMA, including a standardized (-gk2 option) kinship matrix in order to control for population structure among our rhizobia strains. We used the LD-filtered variants to construct k-matrices for each genomic region separately (i.e., one for each of the chromosome, pSymA, and pSymB), given that each replicon exhibits its own distinct evolutionary history (Riley *et al.* 2021).

Although GEMMA calculates p-values for each association, these are likely to be highly conservative once corrected for multiple tests, and do not take into account differences in trait distributions or LD patterns unique to each dataset (Churchill and Doerge 1994; Epstein *et al.* 2018). Thus, in order to determine the significance of each association, we conducted a permutation method similar to Epstein *et al.* (2018), whereby we randomized phenotypes with respect to genotypes, re-ran the association analyses in GEMMA using the same parameters, and repeated this process 1000 times to produce a null distribution of effect sizes for each variant in the model. We then used the findinterval method in R to determine whether our observed effect sizes exceeded the 2.5% or 97.5% intervals of the null distribution, and if so, tagged these variants as being significantly associated with our traits of interest. After running permutation tests for all 10 host-trait combinations, 2,471 (~30%) of the ~8,650 LD-filtered variants were tagged as being significantly associated with one or more traits, in one or more host lines: in total, only 96 of these variants were found on the chromosome, while 998 and 1,377 were found on pSymA and pSymB, respectively. We then determined whether variants were synonymous versus nonsynonymous using SnpEff (v4.3t, Cingolani *et al.* 2012), and extracted functional information for each gene tagged by significant variants using the intersect option in bedtools (v2.29.2, Quinlan and Hall 2010), including the genome annotation file available from NCBI for the reference sequence *Ensifer meliloti* USDA 1106 (assembly ID: GCA_002197065.1). We then identified pleiotropic variants as those that were significantly associated

with more than one trait on the same host line. When variants were significantly associated with multiple rhizobium fitness proxies only, they were designated as underlying rhizobium fitness pleiotropy, whereas when they were associated with at least one rhizobium and plant fitness proxy, they were designated as underlying symbiotic pleiotropy.

Genomic analyses

In order to determine whether pleiotropic variants had discordant or concordant effects on pairwise trait combinations, we conducted correlations of effect sizes calculated in GEMMA for each pairwise trait combination measured on each plant line separately (e.g., nodule number versus nodule weight on DZA) using linear regression (lm function) in base R. When effects were the same in sign (either +,+ or -,-), we tagged them as being concordant, whereas when they were opposite in sign (either +,- or -,+), we tagged them as being discordant. To simplify these results, we plotted the absolute values of each effect size, and mirrored the x-axis so that all points falling to the right or left of zero were concordant or discordant variants, respectively. However, we also present the full regressions in the Supp. Mat. Figs. 1 & 2.

To test whether selection acted on genes underlying rhizobium fitness and symbiotic pleiotropy, we used the R package PopGenome (v2.7.5, Pfeifer *et al.* 2014) to compute several statistics that are commonly used to test for signatures of historical selection and/or departures of neutrality, namely, nucleotide diversity (i.e., π), Tajima's D, as well as Fu and Li's F and D for several subsets of genes: all genes on the pSymA or pSymB plus one on the chromosome (N = 3,103; "all" genes), genes tagged by variants included in our GWAS (N = 1726; "GWAS" genes), genes containing variants significantly associated with one or more traits (N = 1051; "sig" genes), and our focal genes of interest, which contain at least one pleiotropic variant underlying rhizobium fitness or symbiotic pleiotropy (N = 198 total: for rhizobium fitness, N = 10 with concordant and 85 discordant effects; for symbiotic pleiotropy, 97 with concordant and 21 with discordant effects). Test statistics calculated for all 3103 genes appear in Supp. Mat. File S1. We only included genes on the symbiosis plasmids given that only one of our focal genes was found on the chromosome, whereas 93 were found on pSymA and 104 on pSymB. In order to control for unequal sampling sizes, we conducted t-tests in which a random subsample of equivalent size to our focal genes was conducted, p-values recorded, and then repeated 1000 times to calculate the proportion of tests in which our focal and randomized gene subsets were not significantly different (i.e., $P > 0.05$). If the proportion of non-significant tests was less than 10%, then we considered the focal gene category to be significantly different from the randomized set. Because we found that both "GWAS" and "sig" genes had elevated values of diversity compared to the entire genome (Supp. Mat. Table S1), likely because these genes had to contain variants in order to be included in our GWAS, we decided to take a conservative approach by comparing our focal genes of interest to the "sig" genes as our null.

Data Availability

Strains and plant lines are available upon request. All code will be made available on github upon publication. The Supplementary Materials doc contains additional figures and tables, as well as descriptions of files that can be downloaded from bioRxiv directly. Once raw sequence reads and assemblies are archived and made available on NCBI, accession numbers will be included here as well.

Results

Correlations amongst rhizobium fitness proxies differed between proxy combinations and host genotypes

Tradeoffs at the organismal level, plus underlying antagonistic pleiotropy at the genomic level, suggest that the genetic mechanisms rhizobia use to form more nodules in single inoculation make them less able to proliferate within and compete for nodules in mixed inoculation (Fig. 1). For some proxies, but not others, these patterns depended on which host genotype rhizobia are interacting with. First, in single inoculation, when we regressed strain means for nodule number and nodule weight, we found significant negative correlations for both hosts (Fig. 1A left), indicating that strains creating larger nodules tended to form fewer total nodules on both host genotypes. At the genomic-level, most significant and all pleiotropic variants had discordant effects (Fig. 1A right), similarly indicating a tradeoff whereby variants that are positively associated with nodule number tend to be negatively associated with nodule weight, or vice-versa.

Comparing single and mixed inoculation environments, when we regressed strain means for competitive fitness and nodule number, we found a weak but significant negative relationship for DZA only (Fig. 1B left), indicating that strains that were more common in mixed inoculation formed fewer nodules in single inoculation. At the genomic-level, most variants had discordant effects (Fig. 1B right), again indicating a tradeoff between proxies at this scale. For A17, there was no relationship between these fitness proxies at the organismal-level (Fig. 1B right) whereas at the genomic-level, although a similar proportion of significant variants had discordant and concordant effects, most pleiotropic variants had discordant effects (Fig. 1B right).

We found a significant, positive relationship between strain means for competitive fitness and nodule weight, again for DZA only (Fig. 1C left). This result indicates that strains that were more commonly found in nodules in mixed inoculation formed larger nodules in single inoculation. At the genomic-level, variant effects also tended to be concordant (Fig. 1C right), meaning that variants having positive or negative effects on nodule weight also tended to have consistent (i.e., positive or negative) effects on competitive fitness. For A17, while we found a lack of significant genetic correlation for these fitness proxies (Fig. 1C left), at the genomic level, a greater proportion of significant and all pleiotropic variants had discordant effects on nodule weight and competitive fitness (Fig. 1C right), suggesting a potential trade-off between these traits at the genomic-level. However, we found fewer total pleiotropic variants underlying the relationship between competitive fitness and nodule weight (Fig. 2A, right) compared to the other two proxy pairs, suggesting that these proxies are largely governed by different molecular mechanisms and likely evolve independently, especially on A17.

Alignment between host fitness and several rhizobial fitness proxies

We found mostly concordant relationships between competitive fitness and plant fitness at both the organismal and genomic levels (Fig. 2B,C), suggesting that conflict among these traits is largely resolved in natural rhizobium populations. We found no correlation, in either host, between strain means for nodule

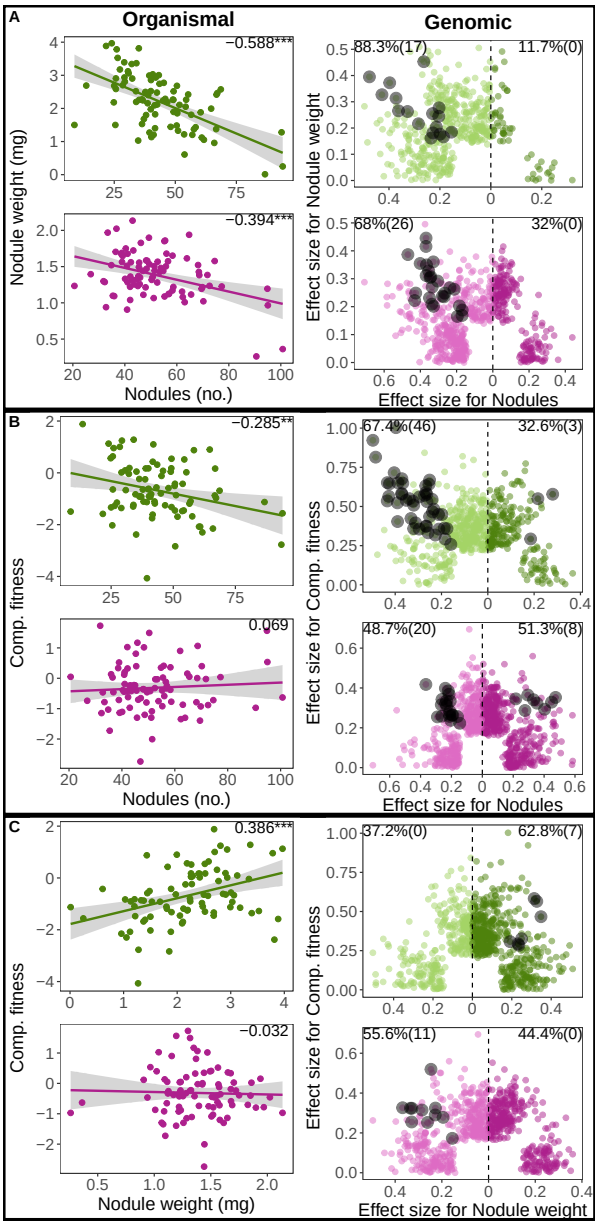

Figure 1 Trade-offs among rhizobia (*Ensifer meliloti*) fitness proxies prevail at both the organismal (left panels) and genomic-levels (right panels). Organismal: Genetic correlations between pairwise fitness proxies correspond to traits measured on plant lines DZA (green, top rows) or A17 (purple, bottom rows), based on 89 *Ensifer meliloti* strains. Dots represent estimated marginal means for nodule number and nodule weight, both being measured in single inoculation experiments, or medians for competitive fitness measured in mixed-inoculation experiments. Numbers at top right of each correlation represent Pearson correlation coefficients, while asterisks represent significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. **Genomic**: Dots represent variant effect sizes (i.e., beta values calculated in GEMMA), whereby effects were regressed for each pairwise trait combination. Dots falling to the right of zero represent variants with concordant (i.e., same-sign) effects, while those falling to the left of zero represent variants with discordant (i.e., opposite-sign) effects. Coloured dots represent variants that were significantly associated with one of the two proxies, while black dots represent pleiotropic variants, i.e., significantly associated with both proxies. Percentages outside of and numbers within parentheses at the top left and right of each plot represent the proportion of total significant variants or pleiotropic variant counts falling to the left or right of zero, respectively.

number and shoot biomass (Fig. 3A left), or nodule number and leaf chlorophyll A content on A17 (Fig. 3D left). However we saw a significantly negative correlation between nodule number and chlorophyll A for DZA (Fig. 3D left). Assuming chlorophyll A indicates the N-fixation efficiency of each strain (Bullock and Anderson 1998; Swiader and Moore 2002; Weese *et al.* 2015), this suggests that strains forming more numerous (and smaller) nodules tended to fix less N. At the genomic-level, most variants had concordant effects for nodule number and shoot biomass on DZA, whereas the opposite was true for the same trait pair on A17 (Fig. 3A right). Most variants had discordant effects on nodule number and chlorophyll A content on both hosts as well (Fig. 3D right).

Strain means for nodule weight and shoot biomass were significantly positively correlated for both hosts (Fig. 3B left). Nodule weight and chlorophyll A were significantly positively correlated on DZA, but not on A17 (Fig. 3E left). At the genomic-level, most significant and all pleiotropic variants had concordant effects for nodule weight and shoot biomass (Fig. 3B right), as well as nodule weight and chlorophyll A (Fig. 3E right), on both hosts.

Finally, competitive fitness and shoot biomass were significantly positively correlated for DZA but not for A17 (Fig. 3C left). In contrast, competitive fitness and chlorophyll A content were significantly positive on both hosts (Fig. 3F left). At the genomic-level, most variants had concordant effects on competitive fitness and shoot biomass (Fig. 3C right), and this pattern was even stronger between competitive fitness and chlorophyll A on DZA with all but one pleiotropic variant having concordant effects (Fig. 3F right). In contrast, variant effects were equally mixed on A17 for competitive fitness and chlorophyll A (Fig. 3F right).

Selection acts differently on genes underlying alignment versus conflict

Our GWAS approach not only allows us to identify the genes associated with fitness conflict or alignment, but to then go a step further and test whether these focal genes show signatures of selection or other processes that would cause patterns of molecular evolution to deviate significantly from neutrality. We *a priori* expected genes underlying fitness conflict to be higher in diversity; instead, our results using four metrics for diversity suggest the opposite – that genes underlying alignment show stronger signatures of balancing selection. For genes underlying both categories of rhizobium fitness pleiotropy (i.e., concordant and discordant, Fig. 4A) as well as genes underlying discordant symbiotic pleiotropy (Fig. 4B), none had elevated Pi, Fu and Li's F, and Fu and Li's D relative to the null expectation (all comparisons > 0.1). Genes underlying concordant symbiotic, however, tended to have elevated statistics relative to null expectation (Fig. 4B), for three of the four test statistics (π, $p = 0.039$; Fu and Li's F, $p = 0.056$; Fu and Li's D, $p = 0.001$), suggesting that genes underlying fitness alignment tend to be higher in nucleotide diversity and show stronger signatures of balancing selection.

Variants with pleiotropic effects on rhizobium fitness proxies share little overlap with those underling symbiotic pleiotropy

Determining and comparing the genetic basis of pleiotropy expressed at multiple biological scales (i.e., within an individual versus across multiple individuals engaged in symbiosis) can reveal whether the genetic architecture of pleiotropy is governed by the same molecular mechanisms regardless of scale,

or whether pleiotropy at each scale is evolutionarily distinct, and thus, likely to evolve independently. We compared variants associated with multiple rhizobium fitness proxies (i.e., rhizobium fitness pleiotropy) with variants associated with both plant and rhizobium fitness proxies (i.e., symbiotic pleiotropy) and found little genetic overlap at both the variant and gene levels between pleiotropic scales. Specifically, we found a total of 278 variants (Supp. Mat. File S2), 148 of which (∼53%) uniquely underlie symbiotic pleiotropy, 110 (∼40%) uniquely underlie rhizobium fitness pleiotropy, while only 20 (∼7%) were overlapping between the two categories, suggesting that the genetic mechanisms underlying rhizobium fitness versus symbiotic pleiotropy are largely different. Similarly, at the gene-level (i.e., excluding any non-coding genes), we found a total of 198 genes tagged by our pleiotropic variants (Supp. Mat. Table S2), 99 of which (∼50%) uniquely underlie symbiotic pleiotropy, 70 (∼35%) uniquely underlying rhizobium-fitness pleiotropy, and 29 (∼15%) overlapping between the two categories. Within each category (i.e., rhizobium fitness or symbiotic pleiotropy), we similarly found little overlap in variants between host lines. For rhizobium fitness, we found 61 and 56 unique pleiotropic variants on A17 and DZA, respectively, while only 13 were shared, all of which underlied symbiotic pleiotropy as well and can thus be considered "universally pleiotropic". For variants underlying symbiosis pleiotropy, 60 and 93 were unique to A17 and DZA, respectively, while only 15 were shared, 13 of which were the same "universally pleiotropic" variants as described above.

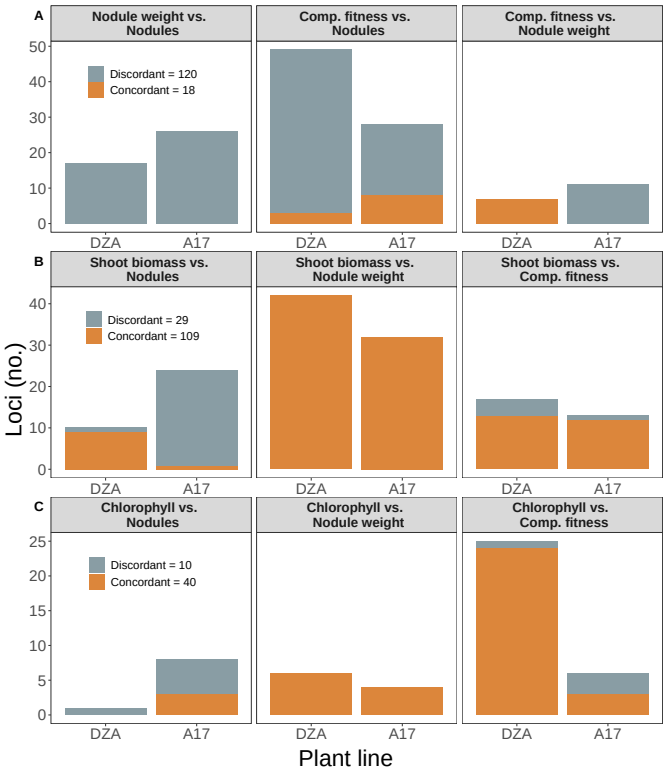

Figure 2 Rhizobium fitness (A) and symbiotic pleiotropy (B,C) dominated by variants with discordant (grey) or concordant (orange) effects, respectively. Variants associated with two or more rhizobium and/or plant fitness proxies are considered to be pleiotropic, counts of which with same sign (i.e., concordant) or opposite sign (i.e., discordant) effects on both traits appear in the leftmost panels in A-C.

Most genes tagged by significant variants at both scales involved biological processes based on UNIPRO keywords including "amino acid transport" and "transcriptional regulation", as well as molecular functions including "oxidoreductase", "transferase", "DNA binding", "lyase", and "hydrolase", suggesting that overlap in gene networks and functions may be more common across pleiotropic scales, which could be more formally tested in follow up transcriptomic studies and gene co-expression network analyses. In subsequent paragraphs, we highlight some of the noteworthy genes identified in our analysis for each pleiotropic category.

For genes underlying rhizobium fitness pleiotropy, we found a particular variant of interest in one of the more well-known symbiosis genes on pSymA, *noeA* (nodulation protein), underlying the tradeoff between nodule weight and nodule number, although only on DZA. We found a strong signal of tradeoffs between nodule number and nodule weight in genes on pSymA involved in catalytic activity (e.g., Fumarylacetoacetate hydrolase), cellular membrane integrity (e.g., *dctQ* TRAP permease), as well as genes involved in oxidoreductase activity (e.g., *thuB*). Several genes contained multiple variants that were associated both with discordant pleiotropy between nodule number and either nodule weight or competitive fitness, as well as concordant pleiotropy between nodule weight and competitive fitness, one of which was the only gene of interest found on the chromosome (i.e., *sndH* L-sorbosone dehydrogenase), while the other two were found on pSymB (e.g., methanol dehydrogenase protein, uncharacterized domain-containing protein).

We found several symbiotic pleiotropic variants in canonical symbiosis genes on pSymA, including *fix*, *nod*, and *nif*, all of which underlie fitness alignment. For example, the major allele of a variant located within the *fixI2* gene was associated with an increase in nodule weight and shoot biomass on DZA, while the major allele of a variant in *nodH* decreased both shoot biomass and nodule weight on A17 (Supp. Mat. File S2). In general, we found a strong signal in branched-chain amino acid transport and many aminotransferases for compounds important in bacteroids and symbiosis including glutamine, aspartate, asparagine, proline, and putrescine, suggesting that there is lots of potential for alignment and conflict in amino acid cycling pathways, mutants of which have been previously demonstrated to produce important symbiotic phenotypes (Dunn 2015). Genes underlying fitness conflict included a fatty acid desaturase, Pyrroline-5-carboxylate reductase, and aldehyde dehydrogenase, all on A17, while a carbohydrate kinase, DNA helicase, and esterase underlied conflict on DZA.

For genes containing variants that were "universally pleiotopic" on both hosts, including those underlying tradeoffs between rhizobium fitness proxies as well as fitness conflict or alignment, the major allele of these variants tended to be associated with a decrease in nodule number, and an increase in other rhizobium or plant fitness proxies. Universal genes of interest included an ABC transporter permease, dihydroxy-acid dehydratase, and a gluconolactonase, all on pSymB, and a *iclR* family transcriptional regulator on pSymA. Finally, we found many pleiotropic variants in genes involved in processes that are not intuitively linked to symbiosis, such as selenocysteine synthase, molybdoterin, conjugative transfer and plasmid stabilization, hemolysin, and siderophores.

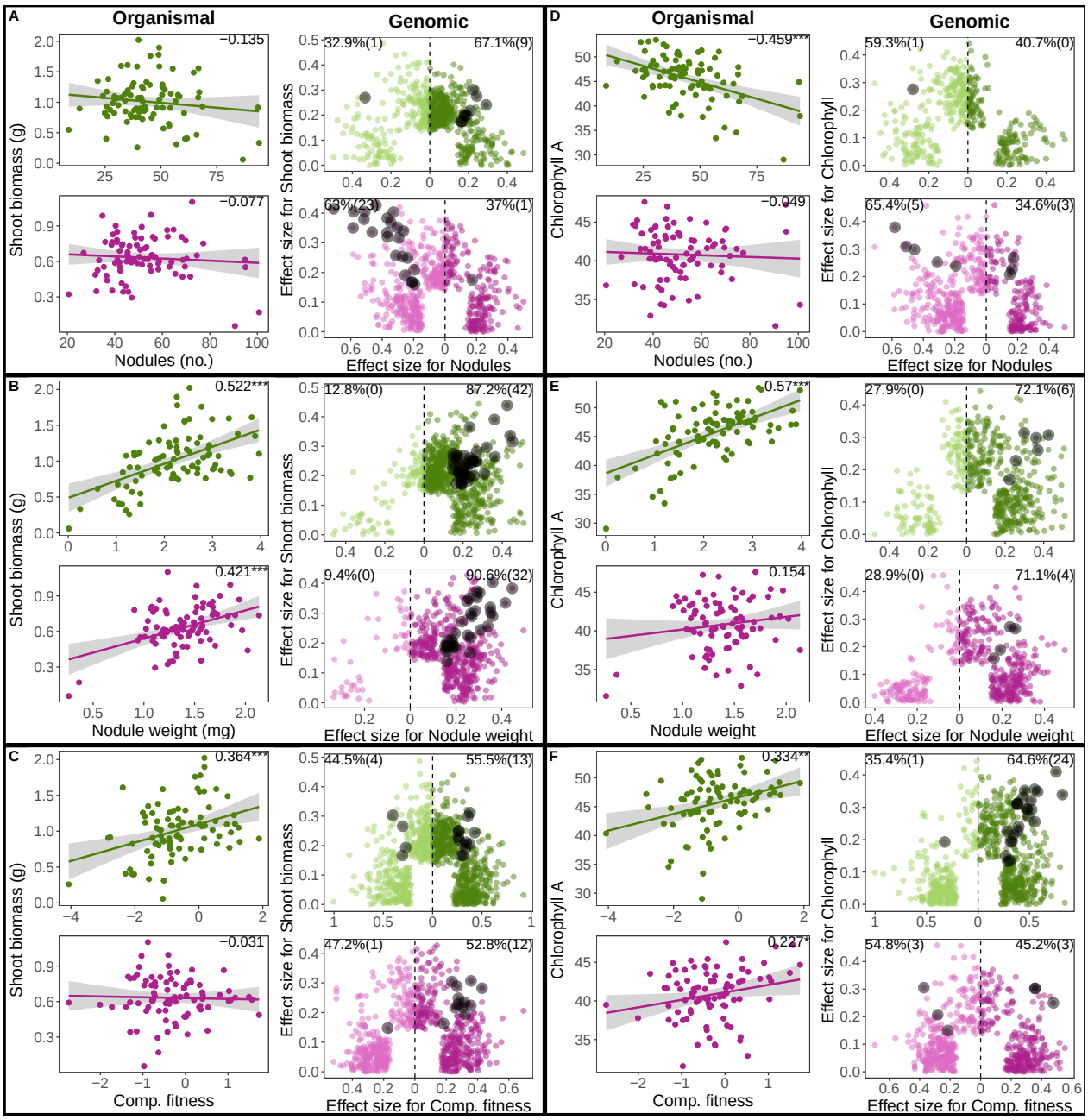

Figure 3 Fitness alignment between rhizobia (*Ensifer meliloti*) and host (*Medicago truncatula*) prevails at both the organismal (left panels) and genomic-levels (right panels). Organismal: Genetic correlations between pairwise fitness proxies correspond to traits measured on plant lines DZA (green, top rows) or A17 (purple, bottom rows), based on 89 *Ensifer meliloti* strains. Dots represent estimated marginal means for shoot biomass (**A-C**) or leaf chlorophyll A content (**D-F**), as well as nodule number and nodule weight, all being measured in single inoculation experiments, or medians for competitive fitness measured in mixed-inoculation experiments. Numbers at top right of each correlation represent Pearson correlation coefficients, while asterisks represent significance: * = P < 0.05; ** = P < 0.01; *** = P < 0.001. **Genomic**: Dots represent variant effect sizes (i.e., beta values calculated in GEMMA), whereby effects were regressed for each pairwise trait combination. Dots falling to the right of zero represent variants with concordant (i.e., same-sign) effects, while those falling to the left of zero represent variants with discordant (i.e., opposite-sign) effects. Coloured dots represent variants that were significantly associated with one of the two proxies, while black dots represent pleiotropic variants, i.e., significantly associated with both proxies. Percentages outside of and numbers within parentheses at the top left and right of each plot represent the proportion of total significant variants or pleiotropic variant counts falling to the left or right of zero, respectively.

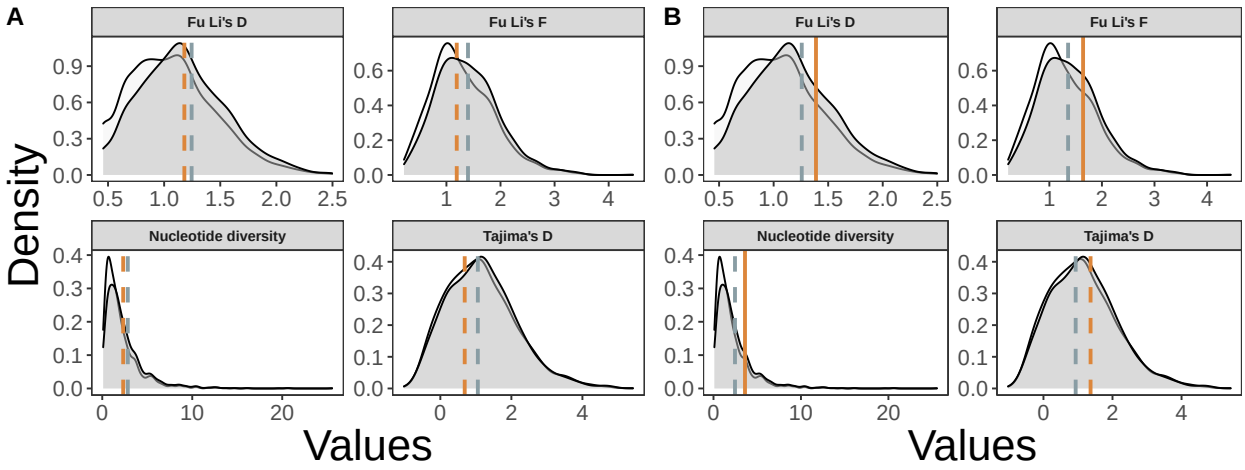

Figure 4 Neutrality statistics show elevated values for genes underlying fitness alignment. Vertical lines represent the average values calculated for four separate statistics, one line for each focal gene category within rhizobium fitness (A) and symbiotic pleiotropy (B), grey for discordant and orange for concordant genes. White and grey distributions represent the same statistics calculated for all genes run through GWAS, and genes tagged as being significant based on GWAS, respectively. Dashed and solid lines represent non-significant ($p > 0.1$) and significant ($p < 0.1$) differences, respectively, between each focal gene category and all significant genes.

Discussion

Recent advancements in studying the genetic architecture underlying symbiotic extended phenotypes gives us the power to address long-standing issues in mutualism evolution with genome-scale resolution. Here, we studied the relationships among multiple proxies of rhizobium fitness and plant fitness, and at both the organismal and genomic levels, to address critical questions about symbiont fitness and ask whether conflict between hosts and symbionts is prevalent across scales. Our results overall suggest that: 1) fitness alignment between hosts and rhizobia is common at both the organismal and genomic-levels, with genes underlying alignment showing elevated nucleotide diversity and signatures of balancing selection; 2) trade-offs between rhizobium fitness proxies (nodule number and size or competitive fitness) exist, but the magnitude of these trade-offs depend on the fitness proxy and host genotype; and 3) the genes underlying correlations among rhizobium fitness proxies (i.e., rhizobium fitness pleiotropy) were largely different than those underlying correlations between host and symbiont fitness proxies (i.e., symbiotic pleiotropy). We discuss these main points in turn below.

Alignment of host and symbiont fitness

Many models of mutualism rely on the key assumption that cooperation is inherently costly and thus that selection should favour less-cooperative, potentially 'cheating', partners that forgo paying the costs whilst continuing to receive benefits – particularly in one-to-many symbioses where a 'tragedy of the commons' (Hardin 1968) should favour less-beneficial symbionts (Bull and Rice 1991; West *et al.* 2002a,b). Using genetic correlations, GWAS, and analyses of nucleotide variation to study the genetic basis of mutualism variation in natural populations of rhizobia, we find little evidence for tradeoffs between rhizobium competitive fitness and plant fitness at the organismal or genomic levels, suggesting strong alignment between host and symbiont fitnesses in nature.

Fitness alignment is ultimately governed by the degree to which mutualistic trait optima are shared among partners, as well as the degree of fitness feedbacks that enforce alignment between hosts and symbionts (Bull and Rice 1991; Sachs *et al.* 2004; Frederickson 2013; O'Brien *et al.* 2021). With respect to nodule number, legume host plants have autoregulation of nodulation to limit the formation of costly nodules once sufficient N levels are achieved (Reid *et al.* 2011). No such constraint exists for rhizobia; every nodule formed is expected to lead to greater potential fitness benefits (Sachs *et al.* 2018). Thus the optimum number of nodules for a plant is likely to be much lower than that for rhizobia. Indeed, the strongest evidence for host-symbiont conflict in our experiment, along with previous studies (e.g., Heath 2010; Ossler *et al.* 2015), comes from regressing plant fitness proxies with nodule number in single inoculation, suggesting conflict over a host's total investment in nodulation. Shared physiological constraints, by contrast, might result in shared fitness optima and align host and symbiont interests. For example, N_2 or O_2 permeability that scales with nodule surface area to volume ratio (King and Purcell 2001), such that nodules too large or small in size might be suboptimal for both partners. Shared constraint could thus explain why we found strong alignment between plant fitness proxies and nodule size; however, a more formal assessment of how selection acts on nodule weight (e.g., cell counts per nodule) would be needed.

In one-to-many mutualisms like legumes and the diverse populations of rhizobia inhabiting their root nodules, fitness feedbacks must operate on a per partner basis to align host and symbiont interests in the presence of conflict. Empirical data supports the presence of such feedbacks via host sanctions wherein nodules fixing more N receive more plant C (Kiers *et al.* 2003; Laguerre *et al.* 2012; Regus *et al.* 2017; Westhoek *et al.* 2021), although the mechanisms are likely not perfectly accurate and have rarely been measured in the wild. Our observations of abundant alignment between host fitness proxies, nodule size, and rhizobium competitive fitness are consistent with fitness feedbacks operating to align host and symbiont fitness in nature, and are particularly striking given that neither host line co-occurs with the rhizobium strains studied, suggesting that feedbacks might operate in diverse communities irrespective of coevolutionary history.

Genomic resolution of conflict and alignment in symbiosis

Using genomics to dig below the phenotypic level can illuminate the mechanisms that favour cooperation over conflict by uncovering the genetic basis of traits central to mutualism evolution in nature, quantifying the extent of symbiotic pleiotropy, and suggesting patterns of historical selection acting on pleiotropic loci. We found little evidence for discordant pleiotropic variants underlying host and symbiont fitness, alongside evidence that selection has acted to maintain this genetic variation through time and/or space. An abundance of concordant pleiotropic variants, like we have found, may have resulted from physiological constraints that make discordant variants impossible (i.e., alleles associated with larger nodules and small host biomass are rare or non-existent), or because of correlational selection that removes discordant variants from the population altogether (Hartl *et al.* 1997; Guillaume and Whitlock 2007; Saltz *et al.* 2017). In addition to direction, the extent of symbiotic pleiotropy (i.e., the number of pleiotropic variants) can inform whether traits are tightly coupled, or could evolve independently. Putting both the degree of concordance/discordance and number of pleiotropic variants together, strong alignment governed by many concordant pleiotropic variants should mean that proxies coevolve in the same direction, whereas conflict governed by many discordant pleiotropic variants should mean that proxies coevolve in opposite directions. Some traits with abundant pleiotropy (e.g., nodule size vs. shoot biomass) suggest a highly polygenic basis governed by many small-effect pleiotropic variants; other traits with little pleiotropy (e.g., nodule number vs. chlorophyll) are largely governed by different sets of genetic mechanisms, and are likely to evolve independently.

One challenge in mutualism research has been identifying patterns of selection acting on traits that determine fitness outcomes in natural populations, particularly for microbial symbioses (Heath and Stinchcombe 2014; Stoy *et al.* 2020). One approach is to study patterns of molecular variation at candidate genes or loci identified through GWAS; key studies to date have often found stabilizing or purifying selection acting on key symbiosis genes for partner recognition or partner benefits, rather than patterns suggesting rapid turnover of alleles underlying conflict (Kimbrel *et al.* 2013; Grillo *et al.* 2016; Yoder 2016; Epstein and Tiffin 2021). In a recent in silico GWAS, O'Brien *et al.* (2021) found that conflict over mutualistic trait optima between hosts and microbes tended to increase genetic diversity due to repeated sweeps, whereas alignment scenarios resulted in stabilizing selection and decreased genetic variation as trait optima were reached. In our empirical dataset, we estimated the direct effects of individual rhizobium alleles on host and symbiont fitness proxies, with the recognition that the "traits" at work are likely the trade of benefits at the molecular level, and found abundant alignment at pleiotropic variants, combined with evidence that these loci harbor particularly high diversity and experience balancing selection to maintain this diversity through time and/or space. While the agents responsible for maintaining diversity remain to be determined, balancing selection acting on genes underlying fitness alignment could maintain quantitative variation in symbiotic outcomes, which has been somewhat of a paradox given that host-driven selection for the "best" symbiont genotype is expected to reduce overall symbiont diversity (reviewed by Heath and Stinchcombe 2014; Stoy *et al.* 2020).

Despite abundant concordance in our data at both levels, our paired approach did reveal several instances of discordance at the variant-level despite alignment at the phenotypic-level (e.g., shoot biomass vs competitive fitness on DZA). These findings highlight the polygenic nature of partner quality variation in nature – where the collective action of individual mutations, their additive and nonadditive effects (Batstone *et al.* bioRiv), and a history of selection shapes the trait variation currently present in natural populations (Conner *et al.* 2004; Lynch and Walsh 1998). Discordant alleles that are "packaged" into concordant multilocus genotypes might be due to linkage disequilibrium resulting from multiple, non-mutually exclusive factors including epistatic interactions among individual variants that render discordant variants effectively neutral and/or past selection acting to favour allelic combinations that increase both host and symbiont fitness (and disfavour discordant combinations; Sinervo and Svensson 2002; Jones *et al.* 2003). We did not detect any evidence consistent with selection having acted on loci containing discordant variants; however, we note that this comparison was underpowered given the small number of variants and does not rule out correlational selection (Svensson *et al.* 2021). Because our experiments were conducted in a single, low N environment, and external N directly reduces the dependence of hosts on rhizobia, we might be underestimating conflict in nature where N sources fluctuate over space and time. How selection on symbiotic mutualisms shifts in natural mutualist populations has rarely been measured (but see Lau *et al.* 2012; Ossler *et al.* 2015; Batstone *et al.* 2020b; Afkhami *et al.* 2021), and should be a priority for future work.

Trade-offs among rhizobium fitness proxies and the rhizobium competition problem

Understanding how microbial symbioses, which are ubiquitous and important in nature, evolve (or coevolve) requires accurate estimates of symbiont fitness in ecologically realistic settings. Given our evidence at both the organismal- and genomic-levels for tradeoffs among proxies, and because diverse strains of rhizobia co-occur in nature, competitive fitness proxies should be used whenever possible (Burghardt *et al.* 2018). Nevertheless these proxies cannot entirely replace single-strain inoculation studies because they cannot be used to assign individual genetic means for whole-plant measures of partner quality (plant biomass and seed number) to individual strains (e.g., Burdon and Thrall 1999; Heath 2010; Epstein *et al.* 2018; Batstone *et al.* bioRiv), necessitating that partner quality and rhizobium fitness be measured on separate individuals.

Comparing even the same fitness proxy across single and mixed inoculation experiments (e.g., nodule number) may be more like comparing apples to oranges if the physiological or signalling processes governing the proxy differ between the two types of experiments. In single-inoculation experiments, plant autoregulation of nodulation (Reid *et al.* 2011) means that plants singly inoculated with a less-effective strain will tend to form more total nodules than if they were inoculated with a more effective N-fixing strain (e.g., Heath 2010; Sachs *et al.* 2010). In mixed inoculation experiments, however, nodule number for a particular strain depends on their competitive ability due to strain-strain competition and/or host signaling interactions (Dowling and Broughton 1986; Wang *et al.* 2018), as well as any pre-infection partner choice, i.e., overrepresentation of more beneficial strains in nodules (Heath and Tiffin 2009; Friesen 2012; Younginger and Friesen 2019; Zhang *et al.* 2020). Therefore, the predominant processes that determine nodule number in mixed inoculation likely differ from those governing nodule number

in single inoculation.

In contrast to nodule number, processes that govern nodule size may be more consistent across single- and mixed-inoculation experiments. For example, if post-infection host control mechanisms (aka host sanctions, *sensu* Heath and Tiffin 2009) represent the proportional allocation of plant C per unit N fixed by rhizobia within a nodule, then nodule size might be indicative of N-fixation efficiency of the strain, perhaps explaining why nodule size and number tend to tradeoff in single inoculation (i.e., plants singly inoculated with less effective N-fixing strains form smaller, more numerous nodules). If nodule size is indeed more consistent across experiment types, we would predict *a priori* to see stronger positive genetic correlations (and a greater genetic overlap) between nodule size and competitive fitness, given that the latter represents not only a strain's ability to compete for nodule occupancy but also for plant C once nodules are formed (Burghardt 2020). Yet, we found evidence for a positive correlation between these proxies only on one of our two host lines (DZA), and very little genetic overlap among variants underlying these proxies overall. Whether N-fixation or C cost (which presumably determine partner quality) also change when strains are in single versus mixed populations on plant roots remains unaddressed, but could be tested using nodule-level measurements (e.g., cell counts per nodule).

Together our results suggest that the genetic architectures underlying rhizobium fitness proxies, and their relationships, are host-dependent, and thus that their evolutionary trajectories are influenced not only by variation in the rhizobium's genome, but also by variation in host traits. For example, host genotypes could differ in their ability to exert sanctions or partner choice, quantitative traits known to vary in host populations (Simonsen and Stinchcombe 2014b; Batstone *et al.* 2017). At the genomic-level, distinct variants underlying discordant pleiotropic effects on one host (A17) and concordant pleiotropic effects on the other (DZA) suggests that different genetic mechanisms (i.e., genes, pathways, metabolic processes) govern the relationship between these traits when rhizobia interact with different hosts. Such host genotype-dependent shifts in the rhizobium genetic architecture of these symbiotic extended phenotypes is supported by transcriptomics studies of G x G interactions (Heath *et al.* 2012; Burghardt *et al.* 2018) and GWAS revealing distinct sets of candidate genes in different host backgrounds (Epstein *et al.* 2018; Batstone *et al.* bioRiv); similar variation exists in hosts (Stanton-Geddes *et al.* 2013) and undoubtedly interacts with the variants we identify here and should be accounted for if we are to build a reliable G→P map.

Genetic basis of rhizobium fitness and symbiotic pleiotropy

Our GWAS approach allows us to identify pleiotropic loci underlying quantitative variation in symbiotic extended phenotypes measured in natural populations, and thus, serves as a powerful starting point to conduct subsequent functional validation and transcriptomic studies, as well as metabolic network reconstructions, that together can be used to build a more reliable G→P map. Many of the loci critical to symbiosis establishment and downstream processes have been identified via functional genomic studies whereby knock-out or knock-down mutants are compared to a reference or wildtype strain, such as *dmi1* in legumes (Ané *et al.* 2004) and *nodD* in rhizobia (Wheatley *et al.* 2020), and are often treated as on-or-off switches for cooperation in models of mutualism theory (e.g., Bull and Rice 1991; West *et al.* 2002a,b; Foster and Kokko 2006). In contrast, our study demonstrates that pleiotropic loci underlying cooperation act more like dials than on-and-off switches. For example, alleles associated with more efficient solute transport across the bacterial membrane might increase the efficacy of N-fixation within the nodule, therefore, benefiting both the rhizobium and plant to a greater extent compared to alleles associated with less efficient transmembrane transport. Indeed, many of the genes we identified as underlying both rhizobium fitness and symbiotic pleiotropy involved analogous processes (e.g., amino acid transport), suggesting that our fine-scale mapping approach could be powerful for uncovering variants that would not otherwise be *a priori* predicted to be important for maintaining cooperation. While our approach allows us to zero-in on particular loci underlying pleiotropy, in silico modelling of both plant and rhizobium (Checcucci *et al.* 2016; Pfau *et al.* 2018; Tesi *et al.* 2020) metabolism that links together genetic information with metabolic pathways could then be used to simulate key symbiotic processes under a wide range of conditions (diCenzo and Finan 2018).

Conclusions

Combining multiple and single inoculation experiments in an association mapping framework using a panel of 191 rhizobium strains from natural populations, we find a lack of conflict underlying mutualism benefits at the phenotypic and genomic levels, and quantify evidence that single-strain and multi-strain environments lead to the expression of distinct traits in rhizobia. While we emphasize the necessity of single strain estimates of partner quality (the effect of a strain on host growth and fitness), our findings nevertheless highlight the need to study the fitness of rhizobia in their population and community contexts. Our results suggest that conflict might be largely resolved in these populations, but paradoxically that this variation is actively maintained by selection, emphasizing the need for rhizobium population biology aimed at identifying the selective agents acting at ecologically relevant scales.

Acknowledgements

For greenhouse help, we thank Alex Riley, Hanna Lindgren, Cassandra Allsup, Laura Goralka, Mannix Burns, Brendan Epstein, and Amy-Marshall Colon, as well as the greenhouse staff, particularly Debbie Black. For feedback on the results and analyses, we thank Peter Tiffin, Brendan Epstein, and Robert Williamson. We acknowledge funding from the National Science Foundation (K.D. Heath: IOS-1645875 and NPGI-1401864; L.T. Burghardt: IOS-1724993 and IOS-1856744), and a Carl R. Woese Institute for Genomic Biology postdoctoral fellowship to R.T. Batstone, strain sequencing by Joint Genome Institute (CSP-1223795).

Literature Cited

Afkhami, M. E., M. L. Friesen, and J. R. Stinchcombe, 2021 Multiple mutualism effects generate synergistic selection and strengthen fitness alignment in the interaction between legumes, rhizobia and mycorrhizal fungi. Ecology Letters .

Ané, J.-M., G. B. Kiss, B. K. Riely, R. V. Penmetsa, G. E. Oldroyd, *et al.*, 2004 *Medicago truncatula dmi1* required for bacterial and fungal symbioses in legumes. Science **303**: 1364–1367.

Barker, D. G., T. Pfaff, D. Moreau, E. Groves, S. Ruffel, *et al.*, 2006 Growing *M . truncatula*: choice of substrates and growth conditions.

Barton, N., 1990 Pleiotropic models of quantitative variation. Genetics **124**: 773–782.

Batstone, R., H. Lindgren, C. M. Allsup, L. A. Goralka, A. B. Riley, *et al.*, bioRiv The genetics of symbiotic extended phenotypes across multiple environments in a model mutualism. Soon to be submitted.

Batstone, R. T., E. M. Dutton, D. Wang, M. Yang, and M. E. Frederickson, 2017 The evolution of symbiont preference traits in the model legume *Medicago truncatula*. New Phytologist **213**: 1850–1861.

Batstone, R. T., A. M. O'Brien, T. L. Harrison, and M. E. Frederickson, 2020a Experimental evolution makes microbes more cooperative with their local host genotype. Science **370**: 476–478.

Batstone, R. T., M. A. Peters, A. K. Simonsen, J. R. Stinchcombe, and M. E. Frederickson, 2020b Environmental variation impacts trait expression and selection in the legume–rhizobium symbiosis. American Journal of Botany **107**: 195–208.

Bronstein, J. L., 1994 Our current understanding of mutualism. The Quarterly Review of Biology **69**: 31–51.

Bronstein, J. L., 2015 *Mutualism*. Oxford University Press, USA.

Bucciarelli, B., J. Hanan, D. Palmquist, and C. P. Vance, 2006 A standardized method for analysis of *Medicago truncatula* phenotypic development. Plant physiology **142**: 207–219.

Bull, J. J. and W. Rice, 1991 Distinguishing mechanisms for the evolution of co-operation. Journal of Theoretical Biology **149**: 63–74.

Bullock, D. and D. Anderson, 1998 Evaluation of the minolta spad-502 chlorophyll meter for nitrogen management in corn. Journal of Plant Nutrition **21**: 741–755.

Burdon, J. and P. Thrall, 1999 Spatial and temporal patterns in coevolving plant and pathogen associations. The American Naturalist **153**: S15–S33.

Burghardt, L. T., 2020 Evolving together, evolving apart: measuring the fitness of rhizobial bacteria in and out of symbiosis with leguminous plants. New Phytologist **228**: 28–34.

Burghardt, L. T., B. Epstein, J. Guhlin, M. S. Nelson, M. R. Taylor, *et al.*, 2018 Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. Proceedings of the National Academy of Sciences **115**: 2425–2430.

Burghardt, L. T., B. Epstein, and P. Tiffin, 2019 Legacy of prior host and soil selection on rhizobial fitness in planta. Evolution **73**: 2013–2023.

Burghardt, L. T., J. Guhlin, C. L. Chun, J. Liu, M. J. Sadowsky, *et al.*, 2017 Transcriptomic basis of genome by genome variation in a legume-rhizobia mutualism. Molecular Ecology **26**: 6122–6135.

Chamberlain, S. A., J. L. Bronstein, and J. A. Rudgers, 2014 How context dependent are species interactions? Ecology Letters **17**: 881–890.

Checcucci, A., M. Bazzicalupo, A. Mengoni, C. Viti, L. Dziewit, *et al.*, 2016 Metabolic modelling reveals the specialization of secondary replicons for niche adaptation in *Sinorhizobium meliloti*. Nature Communications **7**: 1–10.

Churchill, G. A. and R. W. Doerge, 1994 Empirical threshold values for quantitative trait mapping. Genetics **138**: 963–971.

Cingolani, P., A. Platts, L. L. Wang, M. Coon, T. Nguyen, *et al.*, 2012 A program for annotating and predicting the effects of single nucleotide polymorphisms, snpeff: Snps in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly **6**: 80–92.

Conner, J. K., D. L. Hartl, *et al.*, 2004 *A Primer of Ecological Genetics*. Sinauer Associates Incorporated.

Danecek, P., A. Auton, G. Abecasis, C. A. Albers, E. Banks, *et al.*, 2011 The variant call format and vcftools. Bioinformatics **27**: 2156–2158.

Denison, R. F., 2000 Legume sanctions and the evolution of symbiotic cooperation by rhizobia. The American Naturalist **156**: 567–576.

Denison, R. F. and E. T. Kiers, 2011 Life histories of symbiotic rhizobia and mycorrhizal fungi. Current Biology **21**: R775–R785.

diCenzo, G. C. and T. M. Finan, 2018 Techniques for large-scale bacterial genome manipulation and characterization of the mutants with respect to in silico metabolic reconstructions. In *Metabolic Network Reconstruction and Modeling*, pp. 291–314, Springer.

Dos Santos, M., M. Ghoul, and S. A. West, 2018 Pleiotropy, cooperation, and the social evolution of genetic architecture. PLoS Biology **16**: e2006671.

Dowling, D. and W. Broughton, 1986 Competition for nodulation of legumes. Annual Reviews in Microbiology **40**: 131–157.

Dunn, M. F., 2015 Key roles of microsymbiont amino acid metabolism in rhizobia-legume interactions. Critical Reviews in Microbiology **41**: 411–451.

Epstein, B., R. A. Abou-Shanab, A. Shamseldin, M. R. Taylor, J. Guhlin, *et al.*, 2018 Genome-wide association analyses in the model rhizobium *Ensifer meliloti*. MSphere **3**.

Epstein, B. and P. Tiffin, 2021 Comparative genomics reveals high rates of horizontal transfer and strong purifying selection on rhizobial symbiosis genes. Proceedings of the Royal Society B **288**: 20201804.

Ferrari, J. and F. Vavre, 2011 Bacterial symbionts in insects or the story of communities affecting communities. Philosophical Transactions of the Royal Society B **366**: 1389–1400.

Finan, T. M., S. Weidner, K. Wong, J. Buhrmester, P. Chain, *et al.*, 2001 The complete sequence of the 1,683-kb psymb megaplasmid from the n2-fixing endosymbiont sinorhizobium meliloti. Proceedings of the National Academy of Sciences **98**: 9889–9894.

Foster, K. R. and H. Kokko, 2006 Cheating can stabilize cooperation in mutualisms. Proceedings of the Royal Society B **273**: 2233–2239.

Foster, K. R., G. Shaulsky, J. E. Strassmann, D. C. Queller, and C. R. Thompson, 2004 Pleiotropy as a mechanism to stabilize cooperation. Nature **431**: 693–696.

Foyer, C. H., H.-M. Lam, H. T. Nguyen, K. H. Siddique, R. K. Varshney, *et al.*, 2016 Neglecting legumes has compromised human health and sustainable food production. Nature Plants **2**: 1–10.

Frederickson, M. E., 2013 Rethinking mutualism stability: cheaters and the evolution of sanctions. The Quarterly Review of Biology **88**: 269–295.

Frederickson, M. E., 2017 Mutualisms are not on the verge of breakdown. Trends in Ecology & Evolution **32**: 727–734.

Friesen, M. L., 2012 Widespread fitness alignment in the legume–rhizobium symbiosis. New Phytologist **194**: 1096–1111.

Friesen, M. L., 2013 Microbially mediated plant functional traits. Molecular Microbial Ecology of the Rhizosphere **1**: 87–102.

Friesen, M. L. and K. D. Heath, 2013 One hundred years of solitude: integrating single-strain inoculations with community perspectives in the legume–rhizobium symbiosis. New Phytologist **198**: 7–9.

Friesen, M. L., S. S. Porter, S. C. Stark, E. J. von Wettberg, J. L. Sachs, *et al.*, 2011 Microbially mediated plant functional traits. Annual Review of Ecology, Evolution, and Systematics **42**:

23–46.
Friesen, M. L., E. J. von Wettberg, M. Badri, K. S. Moriuchi, F. Barhoumi, *et al.*, 2014 The ecological genomic basis of salinity adaptation in tunisian *Medicago truncatula*. BMC Genomics **15**: 1160.
Galibert, F., T. M. Finan, S. R. Long, A. Pühler, P. Abola, *et al.*, 2001 The composite genome of the legume symbiont sinorhizobium meliloti. Science **293**: 668–672.
Garrison, E. and G. Marth, 2012 Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:1207.3907 .
Gould, A. L., V. Zhang, L. Lamberti, E. W. Jones, B. Obadia, *et al.*, 2018 Microbiome interactions shape host fitness. Proceedings of the National Academy of Sciences **115**: E11951–E11960.
Grillo, M. A., S. De Mita, P. V. Burke, K. L. Solórzano-Lowell, and K. D. Heath, 2016 Intrapopulation genomics in a model mutualist: Population structure and candidate symbiosis genes under selection in *Medicago truncatula*. Evolution **70**: 2704–2717.
Guillaume, F. and M. C. Whitlock, 2007 Effects of migration on the genetic covariance matrix. Evolution: International Journal of Organic Evolution **61**: 2398–2409.
Hardin, G., 1968 The tragedy of the commons. Science **162**: 1243–1248.
Hartl, D. L., A. G. Clark, and A. G. Clark, 1997 *Principles of population genetics*, volume 116. Sinauer associates Sunderland, MA.
Heath, K. D., 2010 Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. Evolution **64**: 1446–1458.
Heath, K. D., P. V. Burke, and J. R. Stinchcombe, 2012 Coevolutionary genetic variation in the legume-rhizobium transcriptome. Molecular Ecology **21**: 4735–4747.
Heath, K. D. and J. R. Stinchcombe, 2014 Explaining mutualism variation: a new evolutionary paradox? Evolution **68**: 309–317.
Heath, K. D. and P. Tiffin, 2007 Context dependence in the coevolution of plant and rhizobial mutualists. Proceedings of the Royal Society B **274**: 1905–1912.
Heath, K. D. and P. Tiffin, 2009 Stabilizing mechanisms in a legume–rhizobium mutualism. Evolution **63**: 652–662.
Hoeksema, J. D., 2010 Ongoing coevolution in mycorrhizal interactions. New Phytologist **187**: 286–300.
Hoeksema, J. D. and E. Bruna, 2015 Context-Dependent Outcomes of Mutualistic Interactions. In *Mutualism*, edited by J. L. Bronstein, pp. 181–202, Oxford University Press, Oxford, UK.
Innocenti, P. and E. H. Morrow, 2010 The sexually antagonistic genes of *Drosophila melanogaster*. PLoS Biology **8**: e1000335.
Jones, A. G., S. J. Arnold, and R. Bürger, 2003 Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. Evolution **57**: 1747–1760.
Jones, E. I., M. E. Afkhami, E. Akçay, J. L. Bronstein, R. Bshary, *et al.*, 2015 Cheaters must prosper: reconciling theoretical and empirical perspectives on cheating in mutualism. Ecology Letters **18**: 1270–1284.
Kessner, D., T. L. Turner, and J. Novembre, 2013 Maximum likelihood estimation of frequencies of known haplotypes from pooled sequence data. Molecular Biology and Evolution **30**: 1145–1158.
Kiers, E. T., W. C. Ratcliff, and R. F. Denison, 2013 Single-strain inoculation may create spurious correlations between legume fitness and rhizobial fitness. New Phytologist **198**: 4–6.
Kiers, E. T., R. A. Rousseau, S. A. West, and R. F. Denison, 2003 Host sanctions and the legume–rhizobium mutualism. Nature **425**: 78–81.
Kiers, T. E., T. M. Palmer, A. R. Ives, J. F. Bruno, and J. L. Bronstein, 2010 Mutualisms in a changing world: an evolutionary perspective. Ecology Letters **13**: 1459–1474.
Kimbrel, J. A., W. J. Thomas, Y. Jiang, A. L. Creason, C. A. Thireault, *et al.*, 2013 Mutualistic co-evolution of typeIII effector genes in *Sinorhizobium fredii* and *Bradyrhizobium japonicum*. PLoS Pathogens **9**: e1003204.
King, C. A. and L. C. Purcell, 2001 Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Science **41**: 1099–1107.
Klinger, C. R., J. A. Lau, and K. D. Heath, 2016 Ecological genomics of mutualism decline in nitrogen-fixing bacteria. Proceedings of the Royal Society B **283**: 20152563.
Laguerre, G., K. Heulin-Gotty, B. Brunel, A. Klonowska, A. Le Quéré, *et al.*, 2012 Local and systemic N signaling are involved in *Medicago truncatula* preference for the most efficient *Sinorhizobium* symbiotic partners. New Phytologist **195**: 437–449.
Lau, J. A., E. J. Bowling, L. E. Gentry, P. A. Glasser, E. A. Monarch, *et al.*, 2012 Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. Acta Oecologica **39**: 80–86.
Lehner, B., 2013 Genotype to phenotype: lessons from model organisms for human genetics. Nature Reviews Genetics **14**: 168–178.
Li, H. and R. Durbin, 2009 Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics **25**: 1754–1760.
Lynch, J. and E. Hsiao, 2019 Microbiomes as sources of emergent host phenotypes. Science **365**: 1405–1409.
Lynch, M. and B. Walsh, 1998 *Genetics and Analysis of Quantitative Traits*, volume 1. Sinauer, Sunderland, MA.
Mackay, T. F. and W. Huang, 2018 Charting the genotype–phenotype map: lessons from the *Drosophila melanogaster* genetic reference panel. Wiley Interdisciplinary Reviews: Developmental Biology **7**: e289.
Mackay, T. F., E. A. Stone, and J. F. Ayroles, 2009 The genetics of quantitative traits: challenges and prospects. Nature Reviews Genetics **10**: 565–577.
McCutcheon, J. P. and N. A. Moran, 2007 Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proceedings of the National Academy of Sciences **104**: 19392–19397.
McCutcheon, J. P. and C. D. Von Dohlen, 2011 An interdependent metabolic patchwork in the nested symbiosis of mealybugs. Current Biology **21**: 1366–1372.
Mitri, S. and K. R. Foster, 2016 Pleiotropy and the low cost of individual traits promote cooperation. Evolution **70**: 488–494.
Moawad, H., W. Ellis, and E. Schmidt, 1984 Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. Applied and Environmental Microbiology **47**: 607–612.
Oono, R., C. G. Anderson, and R. F. Denison, 2011 Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their reproductive clonemates. Proceedings of the Royal Society B **278**: 2698–2703.
Oono, R., R. F. Denison, and E. T. Kiers, 2009 Controlling the reproductive fate of rhizobia: how universal are legume sanctions? New Phytologist **183**: 967–979.

Ossler, J. N., C. A. Zielinski, and K. D. Heath, 2015 Tripartite mutualism: Facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. American Journal of Botany **102**: 1332–1341.

Ostrowski, E. A., Y. Shen, X. Tian, R. Sucgang, H. Jiang, *et al.*, 2015 Genomic signatures of cooperation and conflict in the social amoeba. Current Biology **25**: 1661–1665.

Otto, S. P., 2004 Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proceedings of the Royal Society B **271**: 705–714.

O'Brien, A. M., C. N. Jack, M. L. Friesen, and M. E. Frederickson, 2021 Whose trait is it anyways? Coevolution of joint phenotypes and genetic architecture in mutualisms. Proceedings of the Royal Society B **288**: 20202483.

Parker, M. P., 1995 Plant fitness variation caused by different mutualist genotypes. Ecology **76**: 1525–1535.

Pfau, T., N. Christian, S. K. Masakapalli, L. J. Sweetlove, M. G. Poolman, *et al.*, 2018 The intertwined metabolism during symbiotic nitrogen fixation elucidated by metabolic modelling. Scientific Reports **8**: 1–11.

Pfeifer, B., U. Wittelsbürger, S. E. Ramos-Onsins, and M. J. Lercher, 2014 Popgenome: an efficient swiss army knife for population genomic analyses in r. Molecular Biology and Evolution **31**: 1929–1936.

Pigliucci, M., 2010 Genotype–phenotype mapping and the end of the 'genes as blueprint' metaphor. Philosophical Transactions of the Royal Society B **365**: 557–566.

Porter, S. S. and E. L. Simms, 2014 Selection for cheating across disparate environments in the legume-rhizobium mutualism. Ecology Letters **17**: 1121–1129.

Queller, D. C., 2019 Pleiotropy and synergistic cooperation. PLoS Biology **17**: e3000320.

Quinlan, A. R. and I. M. Hall, 2010 Bedtools: a flexible suite of utilities for comparing genomic features. Bioinformatics **26**: 841–842.

Ratcliff, W. C., K. Underbakke, and R. F. Denison, 2011 Measuring the fitness of symbiotic rhizobia. Symbiosis **55**: 85–90.

Regus, J. U., K. W. Quides, M. R. O'Neill, R. Suzuki, E. A. Savory, *et al.*, 2017 Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. American Journal of Botany **104**: 1299–1312.

Reid, D. E., B. J. Ferguson, S. Hayashi, Y.-H. Lin, and P. M. Gresshoff, 2011 Molecular mechanisms controlling legume autoregulation of nodulation. Annals of Botany **108**: 789–795.

Revelle, W., 2018 'psych': Procedures for Personality and Psychological Research.

Riley, A., M. Grillo, B. Epstein, P. Tiffin, and K. Heath, 2021 Partners in space: Discordant population structure between legume hosts and rhizobium symbionts in their native range. bioRxiv .

Roff, D. *et al.*, 2000 Trade-offs between growth and reproduction: an analysis of the quantitative genetic evidence. Journal of Evolutionary Biology **13**: 434–445.

Sabree, Z. L., C. Y. Huang, G. Arakawa, G. Tokuda, N. Lo, *et al.*, 2012 Genome shrinkage and loss of nutrient-providing potential in the obligate symbiont of the primitive termite *Mastotermes darwiniensis*. Applied and Environmental Microbiology **78**: 204–210.

Sachs, J., J. Russell, Y. Lii, K. Black, G. Lopez, *et al.*, 2010 Host control over infection and proliferation of a cheater symbiont. Journal of Evolutionary Biology **23**: 1919–1927.

Sachs, J. L., U. G. Mueller, T. P. Wilcox, and J. J. Bull, 2004 The evolution of cooperation. The Quarterly Review of Biology **79**: 135–160.

Sachs, J. L., K. W. Quides, and C. E. Wendlandt, 2018 Legumes versus rhizobia: a model for ongoing conflict in symbiosis. New Phytologist **219**: 1199–1206.

Saltz, J. B., F. C. Hessel, and M. W. Kelly, 2017 Trait correlations in the genomics era. Trends in Ecology & Evolution **32**: 279–290.

Searle, S. R., F. M. Speed, and G. A. Milliken, 1980 Population marginal means in the linear model: an alternative to least squares means. The American Statistician **34**: 216–221.

Simms, E. L., D. L. Taylor, J. Povich, R. P. Shefferson, J. L. Sachs, *et al.*, 2006 An empirical test of partner choice mechanisms in a wild legume–rhizobium interaction. Proceedings of the Royal Society B **273**: 77–81.

Simonsen, A. K. and J. R. Stinchcombe, 2014a Herbivory eliminates fitness costs of mutualism exploiters. New Phytologist **202**: 651–661.

Simonsen, A. K. and J. R. Stinchcombe, 2014b Standing genetic variation in host preference for mutualist microbial symbionts. Proceedings of the Royal Society B **281**: 20142036.

Sinervo, B. and E. Svensson, 2002 Correlational selection and the evolution of genomic architecture. Heredity **89**: 329–338.

Somasegaran, P. and H. J. Hoben, 1994 Methods in Legume-Rhizobium Technology. In *Handbook for Rhizobia*, Springer, New York, NY, US.

Stanton-Geddes, J., T. Paape, B. Epstein, R. Briskine, J. Yoder, *et al.*, 2013 Candidate genes and genetic architecture of symbiotic and agronomic traits revealed by whole-genome, sequence-based association genetics in *Medicago truncatula*. PloS one **8**: e65688.

Stearns, F. W., 2010 One hundred years of pleiotropy: a retrospective. Genetics **186**: 767–773.

Stearns, S. C., 1989 Trade-offs in life-history evolution. Functional Ecology **3**: 259–268.

Stoy, K. S., A. K. Gibson, N. M. Gerardo, and L. T. Morran, 2020 A need to consider the evolutionary genetics of host–symbiont mutualisms. Journal of Evolutionary Biology **33**: 1656–1668.

Svensson, E. I., S. J. Arnold, R. Bürger, K. Csilléry, J. Draghi, *et al.*, 2021 Correlational selection in the age of genomics. Nature Ecology & Evolution **5**: 562–573.

Swiader, J. M. and A. Moore, 2002 Spad-chlorophyll response to nitrogen fertilization and evaluation of nitrogen status in dryland and irrigated pumpkins. Journal of Plant Nutrition **25**: 1089–1100.

Taiyun, W. and S. Viliam, 2017 R package 'corrplot': Visualization of a Correlation Matrix.

Team, R. C., 2016 R foundation for statistical computing. R: a language and environment for statistical computing. .

Tesi, M., T. Pfau, A. Mengoni, M. Fondi, *et al.*, 2020 Genome-scale metabolic reconstruction of the symbiosis between a leguminous plant and a nitrogen-fixing bacterium. Nature Communications **11**: 1–11.

Wagner, G. P. and J. Zhang, 2011 The pleiotropic structure of the genotype–phenotype map: the evolvability of complex organisms. Nature Reviews Genetics **12**: 204–213.

Wang, Q., J. Liu, and H. Zhu, 2018 Genetic and molecular mechanisms underlying symbiotic specificity in legume-rhizobium interactions. Frontiers in Plant Science **9**: 313.

Weese, D. J., K. D. Heath, B. T. Dentinger, and J. A. Lau, 2015 Long-term nitrogen addition causes the evolution of less-cooperative mutualists. Evolution **69**: 631–642.

West, S., E. T. Kiers, I. Pen, and R. Denison, 2002a Sanctions and

mutualism stability: when should less beneficial mutualists be tolerated? Journal of Evolutionary Biology **15**: 830–837.

West, S. A., E. T. Kiers, E. L. Simms, and R. F. Denison, 2002b Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proceedings of the Royal Society B **269**: 685–694.

Westhoek, A., L. J. Clark, M. Culbert, N. Dalchau, M. Griffiths, *et al.*, 2021 Conditional sanctioning in a legume–rhizobium mutualism. Proceedings of the National Academy of Sciences **118**.

Wheatley, R. M., B. L. Ford, L. Li, S. T. Aroney, H. E. Knights, *et al.*, 2020 Lifestyle adaptations of rhizobium from rhizosphere to symbiosis. Proceedings of the National Academy of Sciences **117**: 23823–23834.

Wickham, H., 2011 ggplot2. Wiley Interdisciplinary Reviews: Computational Statistics **3**: 180–185.

Wu, D., S. C. Daugherty, S. E. Van Aken, G. H. Pai, K. L. Watkins, *et al.*, 2006 Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biology **4**: e188.

Yoder, J. B., 2016 Understanding the coevolutionary dynamics of mutualism with population genomics. American Journal of Botany **103**: 1742–1752.

Younginger, B. S. and M. L. Friesen, 2019 Connecting signals and benefits through partner choice in plant–microbe interactions. FEMS microbiology letters **366**: fnz217.

Zhang, X., L. Wang, J. Li, R. T. Batstone, and M. E. Frederickson, 2020 *Medicago truncatula* adjusts root proliferation, nodule formation, and partner choice in response to local n heterogeneity. Plant and Soil **450**: 417–428.

Zhou, X. and M. Stephens, 2014 Efficient multivariate linear mixed model algorithms for genome-wide association studies. Nature Methods **11**: 407–409.