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STABILIZING MECHANISMS IN A LEGUME–RHIZOBIUM MUTUALISM

Katy D. Heath[1,2,3] and Peter Tiffin[1]

[1]*Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108*

[2]*E-mail: katy.heath@utoronto.ca*

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Preferential rewarding of more beneficial partners may stabilize mutualisms against the invasion of less beneficial, that is cheater, genotypes. Recent evidence suggests that both partner choice and sanctioning may play roles in preventing the invasion of less-beneficial rhizobia in legume–rhizobium mutualisms. The importance of these mechanisms in natural communities, however, remains unclear. We grew 12 *Medicago truncatula* maternal families with a mixture of three rhizobium strains from their native range for three plant generations and estimated the symbiotic benefits (nodule number and size) conferred to each rhizobium strain. In this experiment, the majority of *M. truncatula* genotypes formed more nodules with more beneficial rhizobium strains, providing evidence for adaptive partner choice. We also found that three generations of symbiosis resulted in an increase in the relative frequency of rhizobium strains that were most beneficial to plants—suggesting that partner choice affects rhizobium fitness. By contrast, we found no evidence that plants differentially rewarded rhizobia postnodulation via sanctioning leading to differences in nodule size. Taken together, our data suggest that plants have evolved to recognize beneficial rhizobial signals during the early stages of symbiosis, and that signaling between plants and rhizobia may be subject to coevolutionary pressures.

KEY WORDS: **Cheater, honest signal, partner choice, plant-microbe, sanctions, symbiosis.**

Theory predicts that mutualisms, or mutually beneficial species interactions, may be evolutionarily unstable because natural selection should favor "cheaters," which receive greater fitness benefits than they confer to their symbiotic partner (Trivers 1971). In the absence of stabilizing mechanisms, these cheaters are expected to sweep through populations and shift otherwise mutualistic interactions towards parasitism. Mechanisms for rewarding beneficial partners, however, are proposed to select for cooperation (Bull and Rice 1991), and may help explain why many mutualisms appear to have been stable for millions of years (reviewed in Bronstein 1994; Sachs et al. 2004). Stabilizing mechanisms can operate either before symbiosis between individuals is formed, as in the attine ant–fungi-actinomycete symbiosis (Mueller et al. 2004; Zhang et al. 2007), or after symbiosis is formed, as in the mutualisms between yucca plants and pollinating yucca moths (Pellmyr and Huth 1994) and host client fish and cleaner fish (Bshary and Grutter 2002).

The mutualism between leguminous plants (Fabaceae) and symbiotic nitrogen-fixing rhizobia originated nearly 60 million years ago, during the early Tertiary (Doyle 1998; Sprent 2007). During symbiosis with plants, rhizobia live in specialized root nodules comprised of plant tissue, where they fix atmospheric nitrogen (N_2) in return for carbon and amino acids from their host plants (Fred et al. 1932; Lodwig et al. 2003). This interaction benefits plants because nitrogen commonly limits plant growth in terrestrial habitats in temperate ecosystems (Vitousek et al. 1997). The economic and ecological importance of these interactions, combined with the ability to manipulate genotypes in both species, makes plants and rhizobia ideal models for answering fundamental questions about mutualism evolution.

Two aspects of the plant–rhizobium mutualism suggest that these interactions should be susceptible to the invasion of cheater genotypes (Denison 2000; Simms and Taylor 2002). First, in every plant generation, the rhizobia are released into the soil

[3]Present address: Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario M5S 3B2, Canada.

environment from senescent root nodules. In the soil, rhizobia live as saprobes until emerging seedlings find them and symbiosis is reinitiated. Unlike vertical transmission, in which symbionts are passed directly from parent to offspring, such horizontal transmission decouples the reproductive interests of partners, making the mutualism more susceptible to cheaters (Yamamura 1993; Sachs and Bull 2005). Rhizobia, because they can live and reproduce in the soil free from plant hosts, may experience selection that favors traits detrimental to plant hosts. Second, rhizobium populations are diverse, and individual plants are commonly found in symbiosis with many genotypes ("strains")—introducing a potential "tragedy of the rhizobial commons," in which the best rhizobial strategy is to hoard benefits while others cooperate (Denison 2000; West et al. 2002).

Context-dependent selection resulting from plant genotype × rhizobium genotype interactions or environmental heterogeneity may slow the invasion of cheaters, but is unlikely to be strong or consistent enough to maintain cooperation (Heath and Tiffin 2007; K. D. Heath, unpubl. ms.). Additional mechanisms, therefore, likely play a role in preventing the fixation of cheaters in the legume–rhizobium system. One possible mechanism is differential provisioning of fewer resources to nodules that contain less-beneficial rhizobia; that is "sanctioning" (Denison 2000; West et al. 2002). Kiers et al. (2003, 2006) showed that soybean (*Glycine max*) plants are physiologically capable of sanctioning nodules that are experimentally prevented from fixing much N_2. More recently, Simms et al. (2006) found that natural populations of *Lupinus arboreus* reward more cooperative *Bradyrhizobium* isolates with larger nodules, which result in greater proliferation of the rhizobia inside.

Legume hosts may also prevent the invasion of cheater rhizobia by discriminating against less-cooperative rhizobia before forming N_2-fixing nodules, that is "partner choice" (Bull and Rice 1991). For plants, discriminating among rhizobium strains before symbiosis would likely be less costly than sanctioning, because nodule formation requires diverting resources from growth (Layzell et al. 1981). Agricultural systems provide numerous examples of segregating alleles that determine whether plants form nodules with different rhizobium strains (reviewed in Parker 1999). Nevertheless, examinations of plant preference for rhizobia from agricultural fields (reviewed in Dowling and Broughton 1986; Triplett and Sadowsky 1992), or for wild-type or mutant nonfixing strains (e.g., Amarger 1981, Hahn and Studer 1986), have found little evidence that plants can reliably differentiate a priori among more or less cooperative rhizobia. However, these studies typically involve plant and rhizobium genotypes that do not share a coevolutionary history. If plants are able to discriminate among rhizobium genotypes prior to nodule formation, then the intricate signaling process between plants and rhizobia (Perret et al. 2000; Oldroyd and Downie 2004) is likely subject to strong coevolutionary pressure. Therefore, data from agricultural systems may not accurately reflect the evolutionary pressures that mutualistic partners impose upon one another in natural systems.

The efficacy of both sanctioning and partner choice rests on the assumption that nodule size and nodule number are important predictors of rhizobium reproductive fitness. There is some empirical support for nodule size being positively correlated with the number of viable rhizobia inside the nodule (Kiers et al. 2003; Simms et al. 2006; Heath and Tiffin 2007). Nevertheless, how nodule size affects rhizobial genotype frequencies in the soil is unknown. There is stronger support that rhizobia benefit from nodulation: rhizobium population sizes have long been known to increase after senescence of fields containing compatible, versus incompatible, legume hosts (Kuykendall 1989; Wilson 1926), and in the soil near roots of compatible hosts (Moawad et al. 1984). However, despite decades of research on rhizobium ecology, it remains to be demonstrated that nodulation per se results in increased frequency relative to other strains in the soil (Simms and Taylor 2002) and, perhaps more importantly, the relative effects that nodule number versus nodule size have on the genotypic frequencies in rhizobium populations remain unclear.

We used a four-generation experiment with the annual legume *Medicago truncatula* (barrel medic) and its symbiont *Sinorhizobium meliloti* from natural communities to investigate the potential for plant genotypes to impose selection for rhizobium cooperation via partner choice or sanctions. The first generation of plants, which represented 12 maternal families sampled from the natural range, was inoculated with an equal mixture of three rhizobium strains (also sampled from the natural range). We grew two more generations of the plant genotype in each pot, then used a single plant genotype in all pots in the fourth generation to sample rhizobia from the soil. We used data from the first generation of plants to test whether (1) plants grown with a mixed population of rhizobia form more nodules with more beneficial strains (i.e., partner choice), (2) plants provide more resources via larger nodules to more beneficial strains (i.e., sanctioning), and (3) there is genetic variation within and/or among plant populations for these mechanisms. We used data on the proportion of nodules inhabited by each strain in the fourth generation to estimate whether differences in nodulation preference among plant genotypes in the first three generations affected the relative frequencies of strains in the soil populations. In other words, these data allowed us to determine whether rhizobia directly benefit from nodulation with plants, and whether these benefits are affected by plant genotype.

Methods

Medicago truncatula, a short-lived annual native to the Mediterranean, and *S. meliloti*, one of two rhizobium species commonly found in symbiosis with *M. truncatula*, is a model system for

investigating legume–rhizobium symbiosis (e.g., Young et al. 2005). We grew 15 plants from each of 12 *M. truncatula* maternal families (four maternal families from each of three populations, *Châteauneuf*, *Nautique*, and *Salses* II, in their native range in southern France) with a mixed community of three rhizobium genotypes that we sampled from the same populations. The four maternal families from each population were haphazardly chosen from a collection made in 2004 (detailed in K. D. Heath, unpubl. ms.). Because of the high ($\sim$ 97.5%) selfing rate in *M. truncatula*, the offspring of maternal plants are expected to be nearly identical genetically (Bonnin et al. 1996a). Nevertheless, the seeds we used came from plants that had been selfed for a generation in the greenhouse to decrease any maternal environmental effects. We inoculated each plant with a mixture of three *S. meliloti* strains, one from each of the geographic locations where plants were sampled (*Chat c*, *Naut c,* and *Sals b*, after K. D. Heath, unpubl. ms.; hereafter referred to simply as *Chat*, *Naut*, and *Sals*). A previous experiment showed that the 12 plant families we used varied in their growth and fitness response to these three strains, indicating that the relative value of the three rhizobium strains differs among plant families (family $\times$ strain interaction for fruit number, $P = 0.0012$; Supporting Fig. S1). This significant interaction reflects many changes in the rank order of strains among plant families (Supporting Fig. S1), although pairwise comparisons revealed that rhizobial genotype had a significant effect on plant growth in only four of 36 pairwise comparisons (Tukey's HSD).

Seedlings were sterilized and cold-stratified as described in Heath and Tiffin (2007) then randomized into 656 mL containers (Stuewe and Sons Inc., Tnagent, OR) filled with a sterile 1:1 mixture of Turface (Profile Products, LLC, Buffalo Grove, IL) and Sunshine Mix #5, which was used because of its low nutrient charge (Sun Gro Horticulture, Bellevue, WA). Seedlings of the first generation plants were inoculated a single time with 1 mL ($\sim 10^6$ cells) of rhizobium inoculum, comprised of an equal proportion of three rhizobium cultures, each of which was grown individually for 48 h at 30°C in liquid MAG media (van Berkum 1990) then diluted to 10^6 cells per mL (based on OD_{670}) prior to mixing and inoculation. Serial dilution plating of separately grown cultures revealed that the relationship between OD reading and viable cell number is similar for the three strains. Plants were grown in the greenhouse with supplemental lighting (16 h days), and pots were elevated and individually top-watered to minimize contamination among pots. In an accompanying experiment, in which plants were inoculated, grown, and watered using similar methods, we found little evidence for rhizobium contamination (K. D. Heath, unpubl. ms.).

After eight weeks in the greenhouse (when the majority of plants had begun flowering), each plant was carefully removed from the soil and 10 nodules were sampled haphazardly (without respect to size). Nodules were stored at 4°C in 1.5 mL tubes atop silica gel and cotton wool until further analysis. Plants, including remaining nodules, were replaced intact in the original soil and allowed to senesce without additional H_2O for approximately one month. After that time, the senescent aboveground biomass was clipped at the soil surface, and new seedlings were planted into pots without disturbing the senesced root system or soil. To test whether plant genotype would affect the genetic composition of the rhizobium population in the soil, we grew two additional plant "generations" (generations II and III) of the same maternal family using the same methods (except that only the first generation of plants was inoculated).

We assayed changes in the relative frequencies of the three rhizobium strains after three generations with the initial maternal families by growing an individual from a single *M. truncatula* family (*Naut 4*, which had been previously shown to form nodules with all three strains [K. D. Heath, unpubl. ms.]) in each pot and then analyzing the composition of strains in plant nodules (hereafter generation IV). Generation IV plants were germinated, planted, and watered as in prior generations, harvested at flowering, and nodules collected using the same approach we used with generation I plants. Because the same plant family was planted into all pots in generation IV, we attribute differences in the frequency of strains in generation IV nodules to among-plant family differences in the soil populations resulting from the previous three generations of symbiosis. This assay does not provide direct estimates of strain frequencies in the soil, however, as strain frequencies in generation IV nodules are affected by the preferential associations of plant family *Naut 4*.

RHIZOBIUM GENOTYPING

Prior to the experiment, we identified rhizobium strain-specific SNPs in a 500-bp intragenic region on the pSymB megaplasmid (Galibert et al. 2001), and developed a PCR-RFLP assay for genotyping the three rhizobium strains used in this experiment. PCR conditions were 35 cycles of 30 sec at 95°C, 1 min at 50°C, and 2 min at 72°C, forward primer: AATGTCTTCCGGAACAGGCG, reverse primer: GGCCATCGAAACCCTCATT. Ten microliters PCR product was then digested by incubating for 1 h at 37°C with 0.25 mg/mL BSA, 1 $\times$ NEB buffer 2, and 1 unit each of ClaI and BsrBI (New England Biolabs, Ipswich, MA). Bands were separated on a 1% agarose gel at 40 V for 2 h and scored visually.

Before genotyping rhizobia, desiccated nodules were rehydrated for 1 h in water and surface-sterilized by dipping in 100% ethanol followed by 1.5 min in commercial bleach. After sterilizing, we measured nodule length and branch number, crushed the nodule with sterile forceps, and streaked the contents onto solid MAG media. Plates were incubated for two to three days at 30°C, at which time we sampled $\sim$1 μL cells from across the streak of colonies. These cells were washed three times in 1 M NaCl and once in 100% EtOH then dried before extracting DNA by

incubating cells in 16 μL 10 mM Tris-HCL (pH 8) plus 4 μL 1 mg/mL proteinase-K at 55°C for ~15 h. The proteinase K enzyme was then deactivated by incubating at 100°C for 10 min. By sampling the entire streak of colonies, as opposed to a single colony, DNA was extracted from all rhizobia inhabiting the nodule, thereby allowing us to identify whether nodules had been infected by more than one rhizobium strain.

Ten nodules from each of four generations I and IV plants from each initial maternal family treatment were rehydrated to identify the rhizobial genotype inside of the nodule. We identified the rhizobial genotype of 338 nodules from 45 generation I plants (an average of 7.4 nodules from 3.75 plants/maternal family) and 346 nodules from 37 generation IV plants. Loss of viability during storage, as well as occasional problems with DNA extraction, prevented us from genotyping all nodules from all plants. For 12 nodules (< 2%), the presence of four bands on the genotyping gels was indicative of mixed infection by two rhizobium strains (two bands result from the digestion of a single strain); these nodules were excluded from further analyses.

DATA ANALYSIS

We used mixed model analysis of variances (ANOVAs) (PROC MIXED, SAS) to test whether plant populations (fixed) and plant maternal families (nested within population, random) significantly influenced the proportion of each of the three rhizobium strains in the nodules of generation I plants. A significant family effect would be evidence that plant genotypes within populations vary in the proportion of nodules formed with each strain, and a significant population effect would be evidence for genetic variation that is structured among populations. Similar analyses with nodule length and branch number as dependent variables were used to test whether plant families vary for the rewards conferred to rhizobia after symbiosis is established. Nodule length is positively correlated with the number of viable rhizobia inside a nodule (Heath and Tiffin 2007), and each *M. truncatula* nodule branch contains an active meristem and reproductive rhizobium cells (K. D. Health, pers. obs.). All dependent variables were square root transformed.

The ANOVAs test for genetic variation in the proportion and size of nodules formed with each strain, but do not test whether plants preferentially form more or larger nodules with strains that are more beneficial. To test whether plants formed more nodules with more beneficial strains, we tested whether the proportion of nodules containing each rhizobium strain when grown in the mixed inoculum environment was positively correlated with the fitness benefits plants obtained from that strain in a single-inoculum environment. A significant positive correlation would indicate that plants form more nodules with more beneficial rhizobium genotypes. Because the proportions of nodules formed by each of the three strains sum to one for each plant, the proportion of nodules containing each strain is nonindependent. Therefore, in addition to calculating the correlations using all of the data together, we also calculated correlations separately for each strain. To determine whether plants provided more resources to nodules containing more beneficial rhizobium strains, we examined correlations between the strain-specific nodule size (length and branch number) and the benefit that plant genotypes obtained from that strain in a single-inoculum environment—with a positive correlation indicating sanctioning by plants. For both of these analyses, the estimates of plant fitness in a single-inoculum environment came from a previous experiment in which plants were grown in similar conditions (Supporting Fig. S1; K. D. Heath, unpubl. ms.).

Effects of nodule number and size on rhizobium fitness

To understand how nodule phenotypes were related to rhizobium fitness, we tested whether the (1) proportion of nodules, (2) nodule length, and (3) nodule branches for each rhizobial strain in generation I plants were significantly correlated with the proportion of strains in the nodules of generation IV plants. Because all generation IV plants were the same genotype, we assume that among-pot differences in rhizobium strain proportions reflect differences among the soil populations caused by the previous three generations of plants grown in the pots. We do not, however, have direct estimates of strain frequencies in the soil, and our data do not provide estimates of absolute differences in the frequencies of rhizobium strains. To control for biased sampling of strains by the plant genotype (*Naut 4*) used in generation IV (this genotype preferentially sampled strain *Sals*; see results), we calculated the relative nodulation frequency (*RN*) for each rhizobium strain (*i*) in the nodules of each plant family (*j*) as

$$RN_{ij} = nod_{ij} - nod_i,$$

where nod_{ij} is the mean proportion of nodules containing strain *i* for all plants of family *j* in generation IV (i.e., the number of strain *i*-containing nodules, divided by the total number of genotyped nodules on the plant, averaged across all plants in the family), and nod_i is the mean of the proportion of nodules containing strain *i* for all plants in generation IV (across all plant families).

Results

VARIATION FOR NODULATION AND PARTNER CHOICE

ANOVA revealed that *M. truncatula* maternal families differed significantly for the proportion of nodules formed with two of the three rhizobium genotypes (*Naut* and *Sals*, Table 1A). Plant families also differed significantly in the length of nodules containing *Sals* or *Chat* strains (Table 1B). The number of branches per nodule did not, however, differ among plant families for any of the

Table 1. **Mixed model ANOVAs of within- and among-population genetic variation for (A) the proportion of nodules occupied by a strain, and (B) the length of occupied nodules for three *S. meliloti* strains, when *M. truncatula* was grown in symbiosis with an equal mixture of the three strains. For random effects, χ^2 (ln-likelihood ratio) is shown. $^{**}P \leq 0.01$.**

Source	*Naut*	*Sals*	*Chat*
(A) Nodule proportion			
Family (Population)	$\chi^2=8.8^{**}$	$\chi^2=9.4^{**}$	$\chi^2=1.3$
Population	$F_{2,9.55}=2.3$	$F_{2,9.7}=0.82$	$F_{2,9.42}=0.22$
(B) Nodule length			
Family (Population)	$\chi^2=1.3$	$\chi^2=7.8^{**}$	$\chi^2=6.8^{**}$
Population	$F_{2,7.14}=0.77$	$F_{2,5.82}=0.78$	$F_{2,8.9}=1.12$

three strains (all $P > 0.29$). We detected no evidence for significant among-population variation in nodule number (Table 1A), length (Table 1B), or branches (all $P > 0.14$); however, we note that, with only four families per population, we had little power to detect such differences. Nevertheless, characterization of growth and reproductive traits (e.g., size, growth rate, reproductive timing, and fecundity; Bonnin et al. 1996b) and symbiotic traits (i.e., fitness response to different rhizobium strains; K. D. Heath, unpubl. ms.) has also revealed high within-population genetic variation in *M. truncatula*, even though this plant is highly selfing and thus might be expected to harbor greater variation among than within populations (Loveless and Hamrick 1984).

No nodules on any plant from three genotypes (*Chat 1*, *Naut 3,* and *Chat 4*) contained the *Sals* rhizobium strain. Two of these plant genotypes, *Chat 1* and *Naut 3*, also did not form nodules with *Sals* in single-strain inoculations, and as a result produced very few fruits (*Naut 3* produced no fruits, and *Chat 1* produced 75% fewer fruits when grown with *Sals* compared to the mean of 8 other strains). Because these fitness estimates represent plant performance in the absence of symbiosis with rhizobia, as opposed to the fitness effect of the *Sals* strain per se, we excluded these two interactions from analyses of partner choice and sanctions. By contrast, *Chat 4* plants formed nodules with *Sals* in single-strain inoculations; therefore, we included this interaction in our analyses.

Two analyses indicate that plants formed symbiosis more often with more beneficial strains—suggesting adaptive partner choice by the plants. First, we detected a significant and positive correlation between the fitness benefits that plant genotypes received from rhizobium strains and the frequency with which they sampled those strains from the mixed soil population (Fig. 1A). Moreover, this positive correlation was not due to among-strain differences in overall quality, as plant families differed in their response to the three strains (Supporting Fig. S1), and correlations calculated for each rhizobium strain separately were positive for all three strains and significantly positive for *Naut* and *Sals* (Fig. 2). Second, individual plant families preferentially formed nodules with more beneficial strains; seven of 12 families formed the greatest number of nodules with the strain that was most beneficial and the fewest nodules with the strain that was least beneficial, significantly more than expected by chance (2×2 contingency test $\chi^2 = 13.1$, df $= 1$, $P = 0.0003$, compared to the null expectation that 1.7 of 10 families with three strain choices, and 1.0 of two families with two strain choices, should match all choices by chance).

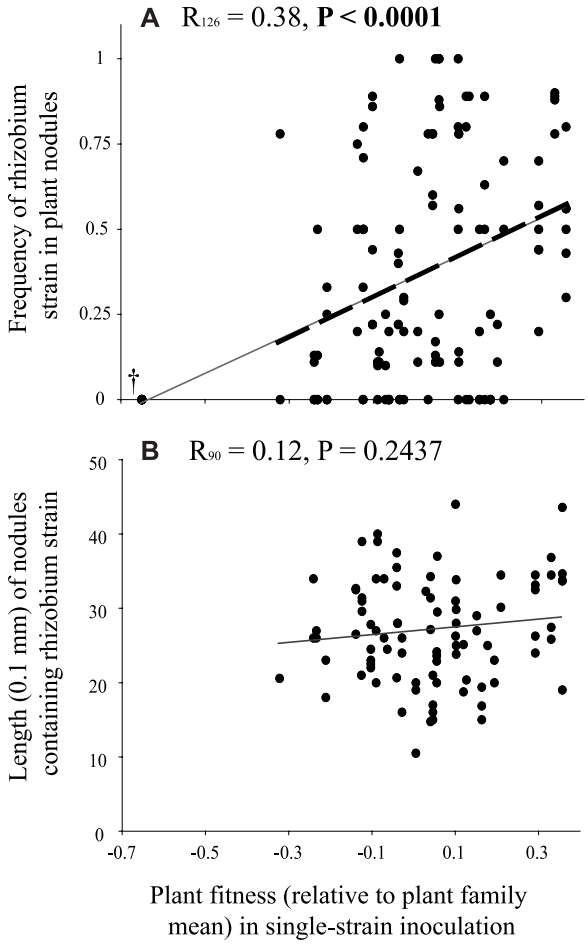

Figure 1. **Tests of partner choice (A) and sanctions (B). Correlation between the proportion of nodules containing a rhizobium strain (A), or the length of nodules containing a strain (B), when plants were grown with a mixture of three strains (vertical axes) and the impact of that strain on plant fitness (relative to the plant family mean) in single-strain inoculation (horizontal axis). †Six observations, all plants in family *Chat 4*, underlie this point. The dotted line in (A) represents the correlation after excluding *Chat 4* observations ($R_{120} = 0.30$, $P = 0.0007$).**

In contrast to differences in the proportion of nodules formed with each strain, strain-specific nodule length, a measure of the resources provided to the nodule by the plant after symbiosis

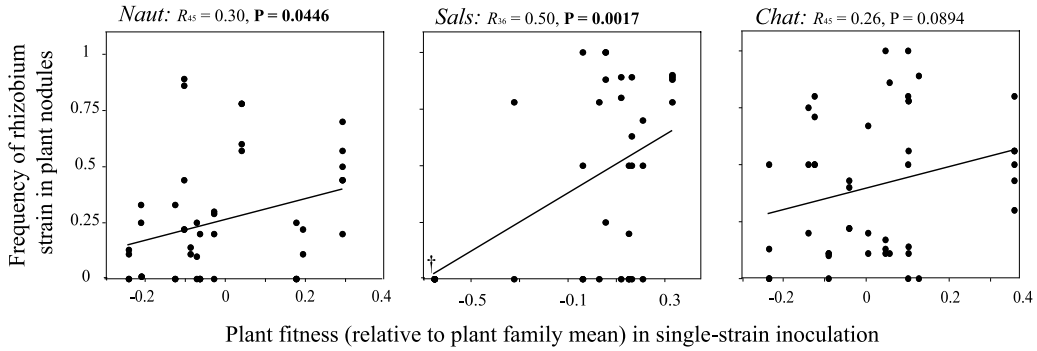

Figure 2. **Partner choice: correlations between the proportion of nodules containing each of three *S. meliloti* strains (*Naut*, *Sals*, or *Chat*) when plants were grown with an equal mixture of the three strains (vertical axis) and the fitness benefits that *M. truncatula* derived from symbiosis with each of the three strains (estimated as mean fruit number of each maternal family when grown with strains individually; horizontal axis). †Six observations underlie this point (plant family *Chat 4*).**

is established, was not significantly correlated with the relative benefits plants obtained from that strain in a single-inoculum environment. This was true whether all data were analyzed together (Fig. 1B), or data from each strain were analyzed separately (all $P > 0.18$). Also in contrast to our analysis of partner choice, in which seven of 12 plant genotypes formed the most nodules with the most beneficial strain and the fewest nodules with the least beneficial strain, only one plant family (*Sals 3*) allocated the most resources to nodules containing the most beneficial strain. This is fewer than expected by chance. We also detected no evidence that nodule branch number was positively correlated with the benefits plants received (combined analyses $R_{90} = -0.12$, $P = 0.28$, individual analyses all $P > 0.12$). These data, therefore, reveal no statistical support for preferential allocation to rhizobium strains postnodulation, as would be expected if sanctions were operating.

THE EFFECTS OF NODULE NUMBER AND NODULE SIZE ON RHIZOBIUM FITNESS

To investigate whether among-plant family differences in partner choice altered the relative frequencies of rhizobium strains in the soil, we assayed strain frequencies after three generations of symbiosis by planting a common plant genotype into each pot (generation IV). For strain *Chat*, the correlation between nodule frequencies in generation I and generation IV plants did not differ significantly from zero (Fig. 3); therefore we found no evidence that the frequency of this strain in nodules of generation IV plants was affected by the plant genotype during the previous three generations. By contrast, the relative frequency of generation IV nodules containing the strain *Naut* was positively correlated with the proportion of *Naut* nodules in generation I plants (Fig. 3)—indicating that *Naut* was more abundant after symbiosis with plant families that formed more nodules with this strain.

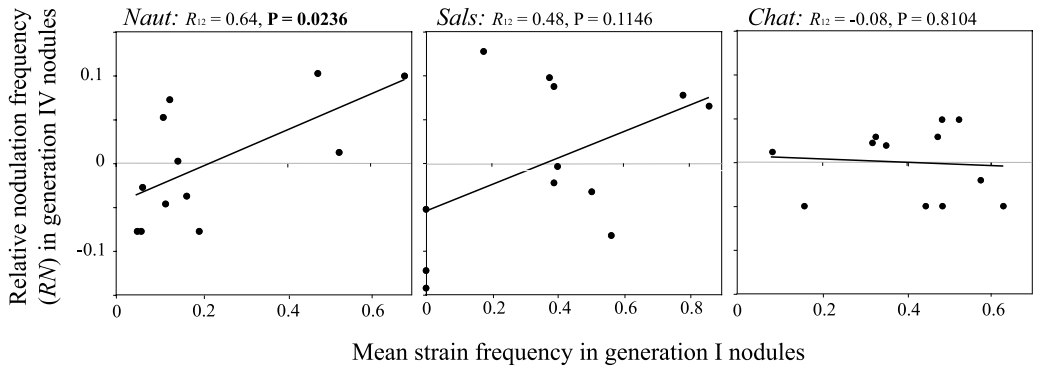

Figure 3. **Rhizobium fitness: correlations between the relative proportion of nodules containing each of the three strains in a common generation IV plant genotype (RN_{ij}, or the deviation from the overall mean of the strain in generation IV; vertical axis) and the frequency of each of the rhizobium strains in the root nodules of 12 *M. truncatula* genotypes in generation I (horizontal axis). Because the same plant genotype was planted in all pots in generation IV, differences in generation IV reflect changes in the relative abundance of strains in soil populations resulting from three prior generations of symbiosis with the 12 different plant families.**

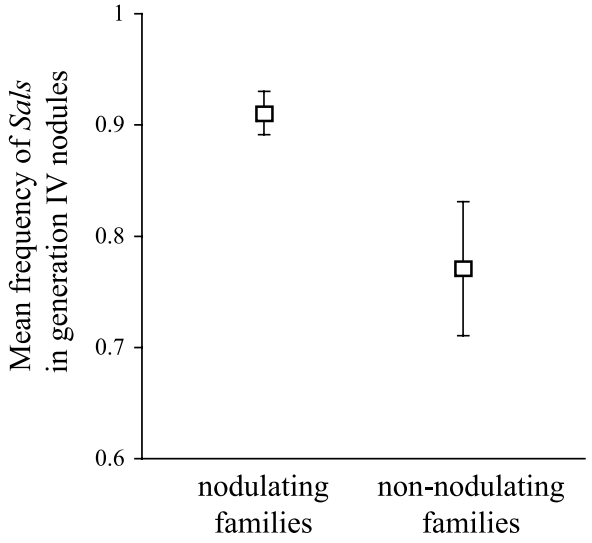

Figure 4. The relative abundance of strain *Sals* in rhizobium populations after evolution with *M. truncatula* families that either did, or did not, form nodules with strain *Sals* ($P = 0.0442$). Shown is the frequency of *Sals*-containing nodules on the common generation IV plant genotype (±SE); therefore, differences among treatments indicate changes in the relative abundance of strains in the soil resulting from three prior generations of symbiosis with different plant families.

Although the relative frequency of *Sals* in generation IV nodules was not correlated with the frequency of *Sals* in generation I nodules (Fig. 3), this strain did provide evidence that nodulation increased rhizobium fitness. In particular, three maternal families (*Chat 1, Naut 3,* and *Chat 4*) did not form nodules with strain *Sals* in this experiment. Pots containing these maternal families, therefore, serve as non-nodulating controls for this strain. If nodulation increases rhizobium fitness, then the frequency of *Sals* in the soils of non-nodulated controls after three plant generations should be lower than the other maternal family treatments. Consistent with this prediction, generation IV plants in *Chat 1, Naut 3*, and *Chat 4* pots made significantly fewer nodules with *Sals* than plants in pots from *Sals*-nodulating plant families ($t = 2.22$, $df = 13.4$, $P = 0.0442$; Fig. 4).

In contrast to the number of nodules occupied, we found little evidence that increases in nodule size resulted in relative increases in strain frequency. All correlations between nodule size (length and branch number) in generation I and the number of nodules occupied in generation IV were nonsignificant (all $P > 0.2$), suggesting that soil rhizobium populations were less affected by nodule size than nodule number.

Discussion

In the absence of selective mechanisms to maintain cooperation, mutualisms should be vulnerable to the invasion of less cooperative (i.e., cheater) genotypes. In this study, we found evidence for adaptive partner choice, which might stabilize mutualisms against the invasion of cheaters and potentially generate positive feedbacks between partners (Law 1985; Law and Koptur 1986). Specifically, we found that the majority of plant genotypes formed the most nodules with the most beneficial rhizobium strains, and the fewest nodules with the least beneficial strains. We also found evidence that nodulation increased rhizobium fitness, as estimated by the relative abundance of rhizobium strains after three generations of symbiosis. Therefore, it appears that *M. truncatula* plants reward more cooperative rhizobium strains by preferentially forming nodules with them. Moreover, the rewarded strains differ among plant genotypes; therefore, the outcomes of rhizobium evolution will depend upon the genetic composition of the plant populations with which they interact.

HONEST RHIZOBIUM SIGNALS AND PARTNER CHOICE

A complex series of signals are exchanged between plants and rhizobia during the initiation, formation, and maintenance of nodules (reviewed in Gage 2004; Jones et al. 2007). Signaling theory, largely developed in the context of intraspecific sexual selection, predicts that "honest" signals (i.e., those that reliably indicate partner quality) may allow for discrimination among potential partners (Dawkins and Krebs 1978; Schaefer et al. 2004). Unlike plants, which are in contact with many rhizobium strains in natural populations, each rhizobium likely has the opportunity to infect only a single root during any growing season. In other words, rhizobia in the soil are not presented with a "market" of potential partners from which to choose (Noë and Hammerstein 1995). Therefore, although plants should experience selection to discriminate among rhizobia of differing quality, rhizobia should experience selection to nodulate with any potential host (given that nodulation increases rhizobium fitness). As such, any honest rhizobium signal that narrows the potential range of plant host genotypes is expected to be selected against.

When selection otherwise favors dishonesty, honest signals are expected to evolve only if the genetic basis of the signal has pleiotropic effects on partner quality (e.g., heavy antlers signal that the male able to carry them is robust), or there is tight linkage between quality and signaling loci (Kirkpatrick 1985; Wolf et al. 1997). Based on current knowledge, however, these conditions are unlikely to be met in *S. meliloti*. The genes expected to affect partner quality in rhizobia are different from those known to act in plant–rhizobium signaling (see Galibert et al. 2001). In fact, *S. meliloti* signaling genes (e.g., *EXO* and *EXP*) are located on a different mega-plasmid from N_2-fixation genes (*NIF* and *FIX*). Even genes located on the same plasmid (e.g., *NOD* and *NIF*) are not necessarily in strong linkage disequilibrium, given that recombination occurs both among- and within-plasmids in natural *S. meliloti* populations (Roumiantseva et al. 2002; Bailly et al.

2006). Of course, it is likely that many genes involved in both partner quality and host-rhizobium signaling have yet to be characterized, and some of these genes may have pleiotropic effects or be in tight linkage. Because selection should favor dishonest rhizobium signaling, as well as the probable lack of linkage disequilibrium between signaling and quality genes, both Denison (2000) and Simms and Taylor (2002) have argued that presymbiotic plant partner choice should be ineffective at preventing the rise of less-cooperative rhizobium genotypes.

Nevertheless, ours is not the first study to find evidence suggestive of adaptive partner choice. For example, some genotypes of soybean from southeast Asia contain the *Rj4* allele, thought to play a role in nod factor perception (Sadowsky and Cregan 1992; Devine and Kuykendall 1996), that excludes many toxin-producing *Bradyrhizobium* strains common to the region but does not prevent nodulation with more cooperative rhizobia (Devine et al. 1990). Suggestive evidence for partner choice also comes from two subspecies of hog peanut (*Amphicarpaea bracteata*): in a natural population, plant lineages were found in symbiosis with the *Bradyrhizobium* strains conferring greater benefit to those lineages (Parker 1995; Wilkinson et al. 1996; Spoerke et al. 1998). Because nodules were sampled directly from a natural plant population, however, the authors could not distinguish the relative roles of adaptive partner choice versus spatial genetic structure in generating the adaptive combinations.

Although we do not know how *M. truncatula* genotypes differentiate among rhizobium strains, rhizobium nod factors (the signals necessary for initiating symbiosis; reviewed in Geurts et al. 2005) may be the signals that plants use to discriminate among strains of differing quality. Two lines of evidence suggest that these signals are good candidates. First is that, as described briefly above, the soybean *Rj4* allele is thought to be involved in nod-factor perception and allows soybeans to discriminate between toxin-producing and beneficial rhizobia (reviewed in Devine and Kuykendall 1996). A second line of evidence comes from analyses of patterns of nucleotide diversity segregating at *M. truncatula* loci known to be important in the perception of rhizobium signals. In particular, an excess of nonsynonymous substitutions suggests that positive selection has driven the divergence of the *NORK* locus (a putative receptor of rhizobium signals, Endre et al. 2002) among *Medicago* species (De Mita et al. 2006). In addition, the *DMI1* locus (likely a mediator of signal reception, Ané et al. 2004) harbors an excess of rare polymorphisms in *M. truncatula*, suggestive of a recent selective sweep (De Mita et al. 2007). As suggested by De Mita et al. (2007), rhizobium signaling and plant perception of signals may coevolve in a scenario similar to host–parasite coevolution. For example, selection may favor plant genotypes that discriminate against less-beneficial strains, much like selection favors plant genotypes that are resistant against common pathogen genotypes (e.g., Burdon and Thrall 1999; Kniskern and Rausher 2006). We are not, however, aware of explicit models or theory predicting how signaling between mutualistic partners coevolves.

SANCTIONS

Although we detected evidence for partner choice, we detected no evidence for plant sanctioning of less-beneficial rhizobia, at least as reflected by decreased nodule size. By contrast, sanctions leading to smaller nodules have been detected in the soybean (*G. max*)–*Bradyrhizobium* (Kiers et al. 2003) and lupine (*L. arboreus*)–*Bradyrhizobium* mutualisms (Simms et al. 2006). Our results are not, however, necessarily in conflict with results from these studies. Kiers et al. (2003) found that plants sanctioned fully formed nodules that were experimentally prevented from fixing N_2 by being placed in an N_2-free environment; therefore, their study shows that sanctioning is physiologically possible, but does not show that sanctioning operates to discriminate among rhizobium genotypes. Moreover, the differences in potential rates of N_2-fixation imposed in that experiment are likely greater than the differences among strains from natural populations. For example, a survey of nine rhizobium genotypes with 12 plant genotypes (K. D. Heath, unpubl. ms.) revealed that, in the vast majority of cases (106 of 108 genotype combinations), even symbiosis with suboptimal rhizobia increased plant fitness compared to uninoculated controls. It seems possible that the differences in rhizobium quality among naturally occurring strains are too small for plants to effectively discriminate. In fact, more recent work by Kiers et al. (2006) found no evidence for sanctions when N_2-fixation was reduced by only 50% compared to controls.

Work by Simms et al. (2006), which revealed evidence for sanctioning but not partner choice, is more similar to ours because they also used plants and rhizobia sampled from co-occurring natural populations. However, they compared nodulation and nodule size of three strains ranked a priori from "poor" to "good" based on their average fitness effect across multiple genotypes of *Lupinus variicolor* and *L. arboreus*. This prescreening may have reduced the likelihood of sampling rhizobium genotypes whose benefit to their hosts was dependent on host genotype. Regardless, the lupine and medic lineages have distinct nodule morphologies and development (Sutton and Paterson 1980) and are estimated to have diverged over 40 million years ago (Lavin et al. 2005)—ample time for the mechanisms mediating host–rhizobium interactions to have diverged. It is possible that the specific mechanisms governing the coevolution of these systems are lineage specific.

Although we detected no evidence for sanctions, it is possible that they were operating, yet our methods prevented us from detecting them. Specifically, because we haphazardly sampled nodules that were visible without magnification, if sanctioning had occurred when nodules were very small, then we may have undersampled sanctioned nodules. We believe this is unlikely, however,

because it is thought that N_2-fixation does not commence until *M. truncatula* nodules are clearly visible to the unaided eye (C. G. Starker, pers. comm.), and because our sample of nodules included those that were very small (0.2–1 mm in length, data not shown). Our methods also would not have detected sanctions if postsymbiotic rewards do not affect nodule size, but only the carbohydrate allocated to the undifferentiated cells in the infection thread (which go on to reproduce outside of the nodule, reviewed in Denison 2000).

RHIZOBIUM FITNESS

Regardless of mechanisms, our data suggest that adaptive partner choice plays a role in enforcing rhizobial cooperation by having a direct effect on rhizobium populations. For example, in generation I plants we found a 10-fold difference in nodulation frequency between plant families that produced the fewest, and those that produced the most, nodules with strain *Naut*. This difference resulted in a ninefold difference in the relative frequency of *Naut* rhizobia detected in generation IV plants (again, between the least- and most-nodulating family treatments). These data are consistent with a large fitness benefit of increased nodule number. Similarly, the *Sals* rhizobium strain was found less frequently in generation IV plants grown in pots in which *Sals*-incompatible, compared to *Sals*-compatible, plant families had been grown for the previous three generations.

Although we found that plant genotype affected the relative frequencies of rhizobium genotypes after three generations of symbiosis, we have no direct estimates of the absolute frequencies of each strain in the soil. In fact, 87% of the nodules we sampled from generation IV plants, all of which were the same plant genotype, were inhabited by the *Sals* strain. In generation I, by contrast, only 50% of the nodules found on this plant genotype were formed with *Sals*. The difference between generations suggests either that *Sals* rhizobia had higher survival or reproduction when growing in the soil, that *Sals* rhizobia had higher reproduction in the plant, or that the conditions in which generation IV plants were grown resulted in preferential nodulation of *Sals* rhizobia by the *Naut 4* genotype. Without direct estimates of frequencies in the soil, we cannot differentiate among the possibilities. If the high frequency with which *Sals* was sampled in generation IV is due to differences in absolute frequencies in the soil, then it would suggest that plant effects on rhizobium strain frequencies via preferential nodulation are smaller than the effects of conditions outside of plant nodules. If true, extrapolating results from microcosm experiments designed to investigate plant–rhizobium coevolution to natural conditions may be misleading.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Fruit number (SE) of 12 *M. truncatula* maternal families with each of the three *S. meliloti* strains in single strain inoculation.

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