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An empirical test of partner choice mechanisms in a wild legume-rhizobium interaction

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Abstract

Mutualisms can be viewed as biological markets in which partners of different species exchange goods and services to their mutual benefit. Trade between partners with conflicting interests requires mechanisms to prevent exploitation. Partner choice theory proposes that individuals might foil exploiters by preferentially directing benefits to co-operative partners. Here, we test this theory in a wild legume-rhizobium symbiosis.

Rhizobial bacteria inhabit legume root nodules and convert atmospheric dinitrogen (N_2) to a plant available form in exchange for photosynthates. Biological market theory suits this interaction because individual plants exchange resources with multiple rhizobia. Several authors have argued that microbial co-operation could be maintained if plants preferentially allocated resources to nodules harbouring co-operative rhizobial strains. It is well-known that crop legumes nodulate non-fixing rhizobia but allocate few resources to those nodules. However, this hypothesis has not been tested in wild legumes which encounter partners exhibiting natural, continuous variation in symbiotic benefit.

Our greenhouse experiment with a wild legume, *Lupinus arboreus*, showed that although plants frequently hosted less co-operative strains, the nodules occupied by these strains were smaller. Our survey of wild-grown plants showed that larger nodules house more *Bradyrhizobia*, indicating that plants may prevent the spread of exploitation by favoring better co-operators.

Key Index Words or Phrases: exploitation, co-operation, symbiosis, mutualism, nitrogen fixation, sanctions

Mutualisms can be modelled as biological markets in which members of each species exchange resources or services (Noë & Hammerstein 1994). However, markets are vulnerable to exploitation and require mechanisms to promote fair commodity exchange (Bronstein 2001). Both "partner choice" and "partner fidelity" can constrain exploitation (Bull & Rice 1991; Noë et al. 1991; Sachs et al. 2004; Simms & Taylor 2002). Partner fidelity occurs when individuals receive returned benefits from their investments in others. Such fitness feedbacks can arise from vertical transmission of symbionts (Axelrod & Hamilton 1981; Fine 1975), certain spatial structures (Doebeli & Knowlton 1998; Wilkinson 1997), or other mechanisms that assure long-term reciprocal interactions. However, positive fitness feedback can be weakened by horizontal transmission and/or competition among potential mutualists (Bronstein et al. 2003; Frank 1994; Frank 1996a; Frank 1996b; Soberon 1985; West et al. 2002a; West et al. 2002b; Wilson et al. 2003). In such cases, exploitation may be constrained by partner choice, in which individuals preferentially extend benefits to co-operative members of the partner species (Bull & Rice 1991; Sachs et al. 2004; Simms & Taylor 2002). There are few empirical tests of the partner choice hypothesis (but see Bshary & Grutter 2002; Grutter & Bshary 2003; Kiers et al. 2003; Mueller et al. 2004).

Root-nodule inhabiting bacteria (hereafter termed rhizobia) are particularly attractive model systems for examining the maintenance of mutualism (Denison 2000; Simms & Taylor 2002). Rhizobia fix atmospheric nitrogen in exchange for photosynthates, but the interaction does not always appear cooperative. Plant and bacteria reproduce and disperse independently (Simms & Taylor 2002) and individual plants usually interact with multiple bacterial genotypes that vary from beneficial to

completely ineffective mutualists (Denison 2000; Moawad & Beck 1991; Moawad et al. 1998; Quigley et al. 1997; Thrall et al. 2000). Several authors have suggested that rhizobial co-operation could be promoted by plant traits that preferentially allocate resources to nodules harbouring co-operative strains (Denison 2000; Simms & Taylor 2002; Sprent 2003; West et al. 2002b). Further, crop legumes are known to sanction (Denison 2000) nodules occupied by ineffective rhizobia (Chen & Thornton 1940; Kiers et al. 2003; Wadisirisuk & Weaver 1985). However, the partner choice hypothesis has not been tested in wild legumes interacting with their naturally occuring rhizobia.

Here, we use a greenhouse experiment to test whether yellow bush lupine, *Lupinus arboreus* Sims, a short-lived perennial shrub in the Fabaceae, can allocate resources to more effective symbionts when nodulated by mixed populations of bacterial strains with which they naturally occur.

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Materials and Methods

Yellow bush lupine is native to the central California coast and is common throughout Bodega Marine Reserve (BMR; 38° 19' 01" N, 123° 04' 18" W). At BMR, *L. arboreus* can occur alone or with up to four other lupine species: *L. nanus, L. bicolor, L. variicolor, and L. chamissonis* (Barbour et al. 1973). Like other lupines (Barrera et al. 1997; Bottomley et al. 1994; Ludwig et al. 1995; but see Stepkowski et al. 2003), lupines at BMR associate predominantly with *Bradyrhizobium* strains that are closely related to the primary soybean symbiont, *B. japonicum*, and are generally designated as *Bradyrhizobium* sp. (*Lupinus*) (Taylor and Simms, unpubl. data).

Rhizobia fix nitrogen only after differentiating into bacteroids within the plant (Simms & Bever 1998). Although there is debate about the ability of bacteroids to survive nodule senescence, de-differentiate, and reproduce, *Bradyrhizobium* bacteroids or vegetative cells of the strain generating bacteriods apparently survive senescence of lupine nodules (Sprent et al. 1987). Mycorrhizae are unlikely to be important for the nutrient or water relations of lupines (Avio et al. 1990; Oba et al. 2001; Trinick 1977).

Seeds and bacteria were collected from six intensively studied sites at BMR (Maron & Simms 2001), three in dunes and three in grasslands, located ca. 500-m apart. Seeds were collected from two maternal plants sampled from each of three sites: South Grassland, Mid Grassland, and Mid Dunes. Ten bacterial isolates were obtained from nodules excised from two naturally occurring lupines at each of five sites: South Grassland, Mid Grassland, South Dunes, Mid Dunes, and Mussel Point.

We genotyped bacterial isolates using PCR-RFLPs of the 16S-23S rRNA internal transcribed spacer (ITS) region (Taylor & Simms, unpubl. data) and characterized their symbiotic benefit to two lupine species, *L. arboreus* and *L. variicolor* (Povich & Simms, unpubl. data). Three strains were selected that differed in RFLP type and represented the observed range of symbiotic benefit to *L. arboreus* (Table 1).

To test whether plants preferentially allocate resources to more beneficial bacteria, we established three mixed inoculation treatments (mediocre and poor), (good and poor), (good and mediocre) and an uninoculated control treatment. Each treatment was applied to one randomly assigned seedling from each of six maternal plants in each of five blocks. Within a block, the six seedlings in an inoculation treatment were each grown in a separate pot, with pots racked together to form a subplot within a

fully randomised split plot design. We maintained one meter distances among racks to prevent cross-contamination. Four racks comprised a block. Plants were assigned to blocks by size, which primarily reflected germination date. Blocks varied by plant age, location on the greenhouse bench, inoculation volume, and harvest date.

On July 25, 2003, seeds for blocks 1 – 3 were scarified and surface sterilised by soaking in concentrated sulphuric acid for 15 minutes and rinsing in sterile deionized water. The seeds were germinated in autoclaved trays of moist vermiculite under metal halide lamps set to a 23-hour photoperiod at room temperature. After 12 days, seedlings were transplanted to sterile Deepots® (Steuwe and Sons) filled with autoclaved calcine-clay (Turface®, Profile Products). On August 6, 2003, seeds for blocks 4 and 5 were surface sterilised for 20 minutes in bleach, rinsed in sterile water, and nick scarified with a razorblade. After imbibing sterile water for three days, germinated seeds were planted directly into sterile Deepots® of autoclaved Turface®. All seedlings were watered four times per day with UV-sterilised carbon-filtered tap water until August 20, 2003, after which they were watered as necessary (usually daily) and fertilised biweekly with nitrogen-free modified Jensen's solution.

Each strain was initiated from a single plated colony grown in liquid culture (MAG media, modified by P. van Berkum from Cole & Elkan 1973) and cryopreserved in 60% glycerol. Inoculant was prepared by regrowing on solid media and washing cells with sterile 0.85M KCl. Cell densities were determined by absorbance and adjusted to 1×10^8 cells ml^{-1}. Cell numbers were calibrated by cytometer and confirmed with plate counts. Mixed inoculants were prepared by combining equal quantities of the two appropriate single-strain inoculants. We pipetted 10 ml of mixed inoculant onto each seedling in blocks 1-3 on August 12, 2003. On September 1, 2003, we inoculated

seedlings in blocks 4-5 with 7 ml. Control plants within a block received the appropriate volume of sterile 0.85M KCl.

Block One was harvested September 21, 2003; remaining plants were harvested January 6 – 7, 2004. During both harvests, we selected a range of nodule sizes from each plant. To ensure that small nodules were not younger than large nodules, we chose small nodules proximal along the roots from which large nodules were obtained. We measured the diameters of six nodules, then each was excised, surface-sterilised with household bleach, and stored at -20ºC in 5X its volume of 20% Chelex 100 resin (Sigma Chemical) in 2X PCR buffer (40mM Tris-HCl, pH 8.0; 100 mM KCl). Immediately after thawing, nodules were heated and vortexed at 95ºC, centrifuged 5 min at 16,000G. The resulting crude extract was stored at -20ºC.

From each sample, we amplified 1440 bp of the intergenic spacer between the large and small ribosomal subunits (hereafter referred to as ITS) using primers ITS450 and ITS1440 (van Berkum & Fuhrmann 2000). The 50uL PCR reaction contained 2.0 mM MgCL, 0.25 mM dNTPs, 0.5 uM primers, 1.25 units *Taq* polymerase (Invitrogen Life Technologies), 1X Invitrogen PCR buffer and 10 uLs of diluted nodule DNA extract (usually a 25-fold dilution). Reactions were initiated with a 2-min 95ºC denaturation and ended with a 10-min extension step at 72ºC. Cycling involved 94ºC denaturation for 30 sec, 70ºC annealing for 40 sec, reduced by 0.5ºC per cycle to 60ºC in the touchdown phase, with an additional 30 cycles at 60ºC, and 72ºC extension for 1.5 min. Aliquots of the PCR product were digested with endonucleases *Mwo*I and *Hga*I. Fragments were separated on 10-cm gels of 1% regular agarose and 2% High Resolution™ agarose (Sigma Chemical) in TAE buffer

at 170 volts for 2-3 hours, stained with ethidium bromide, and visualised on a UV transilluminator.

Due to germination problems, each treatment in block five lacked one plant. Nineteen plants died during a late August heatwave; five were replaced with substitutes of the same age that had been appropriately inoculated contemporaneously. By harvest, 17 additional plants had died, including nine control plants, reducing the number of harvested plants to 85. Of these, one inoculated plant failed to nodulate and PCR reactions failed on all nodules of three other inoculated plants. Ultimately, we obtained informative nodule occupancy data from 65 plants.

Nodule size and strain identity were analysed with a repeated measures nested analysis of variance(ANOVA) with a compound symmetry covariance structure using the SAS 9.1 MIXED procedure (SAS Institute 2004). The model included nodules as repeated measures within plants, plants as random subjects nested within fixed inoculation treatments, and treatments nested within random blocks. Plant family structure was too unbalanced to analyse. The block effect and block by plant(treatment) interaction were non-significant and raised the Akaike Information Criterion score, so these effects were dropped. For each inoculation treatment, we subsequently performed a one-way ANOVA of plant effect on nodule size, used the residuals to classify nodules into two or three size classes, and used chi squared tests to determine whether standardized nodule sizes were distributed independently of strain identity.

To establish the relationship between nodule diameter and *Bradyrhizobium* population size within a nodule, we surveyed six nodules each from 9 *L. arboreus* seedlings at four sites across BMR. Nodules were collected, measured and surface

sterilized as described above. Each nodule was macerated in sterile MAG media and the slurry was serially diluted and plated on MAG-agar plates for colony counts. Plate counts were averaged across at least two replicates per nodule to estimate the *Bradyrhizobium* population size. Colony number was natural log transformed to normalize residuals and regressed on nodule diameter using JMP 5.1.2 (SAS Institute, Inc.).

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Results

We obtained readable bands from 301 nodules. Banding patterns from two nodules suggested occupancy by more than one isolate. We found no evidence of cross-contamination among treatments. At harvest, no control plants were nodulated and all identified strains matched at least one of the strains with which their host plant had been inoculated.

Nodule sizes within plants were significantly influenced by occupant identity ($F_{2,25} = 16.42$, $p < 0.0001$). Within the average plant, nodules that were occupied by the more beneficial strain were significantly larger (Figure 1, a – c). However, because strain identity interacted significantly with treatment ($F_{1,25} = 10.14$, $p < 0.004$), we also examined the strain effect in each treatment separately.

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Of 24 plants inoculated with both good and poor strains, we detected 11 plants nodulated by both strains. Occupant identity explained a significant component of variation in nodule size within plants ($F_{1,10} = 5.38$, $p = 0.04$), with smaller average size among nodules occupied by the poor strain (Fig 1a). Further, contingency table analysis indicated that strain distribution between nodule size categories differed from

random, with the poor strain occurring more frequently in smaller nodules (Table 2a, $\chi_1^2 = 5.00$, $p < 0.03$). Of the 14 plants in which we detected only one strain, all were occupied by the poor strain.

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Of 22 plants inoculated with both mediocre and poor strains, we found 10 nodulated by both strains. Occupant identity explained a significant component of variation in nodule size within plants ($F_{1,9} = 7.91$, $p = 0.02$), with smaller average size among nodules occupied by the poor strain (Fig 1b). Strain distribution among nodule size categories differed from random (Table 2b, $\chi_2^2 = 12.36$, $p < 0.002$), with the poor strain occurring more frequently in smaller nodules. Of the 12 plants in which we detected only one strain, three contained the poor and nine contained the mediocre strain.

Finally, of 18 plants inoculated with both good and mediocre strains, seven plants were nodulated by both strains and occupant identity explained a significant component of variation in nodule size within plants ($F_{1,6} = 10.48$, $p = 0.02$), with smaller average size among nodules occupied by the mediocre strain (Fig 1c). As in the previous two treatments, strain distribution between nodule size categories differed from random, with the mediocre strain occurring more frequently in smaller nodules (Table 2c, $\chi_1^2 = 5.99$, $p < 0.02$). Of the 11 plants in which we detected only one strain, all were occupied by the mediocre strain.

Among 33 nodules sampled randomly from nine wild-grown *L. arboreus* seedlings collected at BMR, total population size of *Bradyrhizobium* cells in a nodule regressed positively on nodule size ($r^2 = 0.2$, $p < 0.01$).

Discussion

In lupines experimentally infected by divergent pairs of naturally occurring *Bradyrhizobium* strains, nodule size varied with the symbiotic effectiveness of bacterial occupants. On average, nodules inhabited by the less beneficial strain were smaller. In a separate survey of wild-grown *L. arboreus*, larger nodules contained more *Bradyrhizobium* cells, as previously found for soybean nodules (Kiers et al. 2003). These results support the hypothesis that legumes can favour more co-operative rhizobia by manipulating bacterial fitness in the nodule (Denison 2000; Simms & Taylor 2002; Sprent 2003; West et al. 2002b).

Although our experiment does not specifically distinguish between plant or bacterial control of allocation to nodules, we argue that the original hypothesis is more parsimonious. Our data complement the findings of Kiers and colleagues (2003), who found that the fresh weights of soybean nodules deprived of atmospheric dinitrogen were smaller than those of unmanipulated nodules occupied by the same strain on the same plant. They suggested that plants might control nodule size in part by altering oxygen supply. Other mechanisms might also contribute to the effect (Simms & Taylor 2002).

Among the 37 plants in which we found only one strain, 25 had been inoculated with a mix including the good strain, yet we detected this strain in none of these plants. This result corroborates other studies, which have shown that the ability of rhizobia to compete for nodulation may be uncorrelated with symbiotic benefit (Bloem & Law 2001; Hafeez et al. 2001; Triplett & Sadowsky 1992; Vasquez-Arroyo et al. 1998).

For nodule-specific plant responses to constrain cheating effectively, individual nodules must be occupied by single bacterial genotypes (Denison 2000; Denison & Kiers 2004; Kiers et al. 2003; West et al. 2002b). Among the 301 nodules from which we obtained readable RFLP bands were two that showed a banding pattern suggestive of occupancy by more than one isolate. These data suggest that multiply infected nodules are rare.

We believe that our study provides the first evidence that nodule size scales with naturally occurring variation in symbiotic effectiveness of the bacterial occupant. Past workers on agricultural legumes have noted that ineffective strains often result in small nodules (Simms & Taylor 2002), but, with a few notable exceptions (Chen & Thornton 1940; Kiers et al. 2003; Singleton & Stockinger 1983) the phenomenon has rarely been quantified.

In summary, our results suggest that post-infection partner choice could be an important mechanism constraining exploitation by rhizobia in this legume-rhizobium mutualism. We look forward to additional tests of the assumptions of this theory.

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References

Avio, L., Sbrana, C. & Giovannetti, M. 1990 The response of different species of *Lupinus* to VAM endophytes. *Symbiosis* **9**, 321-323.

Axelrod, R. & Hamilton, W. D. 1981 The evolution of cooperation. *Science* **211**, 1390-1396.

Barbour, M. G., Craig, R. B., Drysdale, F. R. & Ghiselin, M. T. 1973 *Coastal Ecology: Bodega Head*. Berkeley, CA: University of California Press.

Barrera, L. L., Trujillo, M. E., Goodfellow, M., Garcia, F. J., Hernandez-Lucas, I., Davila, G., van Berkum, P. & Martinez-Romero, E. 1997 Biodiversity of bradyrhizobia nodulating *Lupinus* spp. *International Journal of Systematic Bacteriology* **47**, 1986-1091.

Bloem, J. F. & Law, I. J. 2001 Determination of competitive abilities of *Bradyrhizobium japonicum* strains in soils from soybean production regions in South Africa. *Biology and Fertility of Soils* **33**, 181-189.

Bottomley, P. J., Cheng, H.-H. & Strain, S. R. 1994 Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recovered from a pasture soil. *Applied and Environmental Microbiology* **60**, 1754-1761.

Bronstein, J. L. 2001 The exploitation of mutualisms. *Ecology Letters* **4**, 277-287.

Bronstein, J. L., Wilson, W. G. & Morris, W. E. 2003 Ecological dynamics of mutualist/antagonist communities. *American Naturalist* **162**, S24-S39.

Bshary, R. & Grutter, A. S. 2002 Experimental evidence that partner choice is a driving force in the payoff distribution among cooperators or mutualists: the cleaner fish case. *Ecology Letters* **5**, 130-136.

Bull, J. J. & Rice, W. R. 1991 Distinguishing mechanisms for the evolution of cooperation. *Journal of Theoretical Biology* **149**, 63-74.

Chen, H. C. & Thornton, H. G. 1940 The structure of "ineffective" condules and its influence on nitrogen fixation. *Proceedings of the Royal Society Biological Sciences Series B* **129**, 208-229.

Cole, M. A. & Elkan, G. H. 1973 Transmissible Resistance to Penicillin-G, Neomycin, and Chloramphenicol in Rhizobium-Japonicum. *Antimicrobial Agents and Chemotherapy* **4**, 248-253.

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

Denison, R. F. & Kiers, E. T. 2004 Why are most rhizobia beneficial to their plant hosts, rather than parasitic? *Microbes and Infection* **6**, 1235-1239.

Doebeli, M. & Knowlton, N. 1998 The evolution of interspecific mutualisms. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8676-8680.

Fine, P. E. M. 1975 Vectors and vertical transmission: an epidemiological perspective. *Annals of the New York Academy of Science* **266**, 173-194.

Frank, S. A. 1994 Genetics of mutualism: The evolution of altruism between species. *Journal of Theoretical Biology* **170**, 393-400.

Frank, S. A. 1996a Host control of symbiont transmission: The separation of symbionts into germ and soma. *American Naturalist* **148**, 1113-1124.

Frank, S. A. 1996b Host-symbiont conflict over the mixing of symbiotic lineages. *Proceedings of the Royal Society of London Series B-Biological Sciences* **263**, 339-344.

Grutter, A. S. & Bshary, R. 2003 Cleaner wrasse prefer client mucus: support for partner control mechanisms in cleaning interactions. *Proceedings of the Royal Society of London Series B-Biological Sciences* **270**, S242-S244.

Hafeez, F. Y., Hameed, S., Ahmad, T. & Malik, K. A. 2001 Competition between effective and less effective strains of *Bradyrhizobium* spp. for nodulation on *Vigna radiata*. *Biology and Fertility of Soils* **33**, 382-386.

Kiers, E. T., Rousseau, R. A., West, S. A. & Denison, R. F. 2003 Host sanctions and the legume-rhizobium mutualism. *Nature* **425**, 78-81.

Ludwig, W., Rosello-Mora, R., Aznar, R., Klugbauer, S., Springs, S., Reetz, K., Beimfohr, C., Brockmann, E., Kirchhof, G., Dorn, S., Bachleitner, M., Klugbuaer, N., Springer, N., Lane, D., Nietupsky, R., Weizenegger, M. & Schleifer, K. H. 1995 Comparative sequence analysis of 23S rRNA from Proteobacteria. *Systematic and Applied Microbiology* **18**, 164-188.

Maron, J. L. & Simms, E. L. 2001 Rodent-limited establishment of bush lupine: field experiments on the cumulative effect of granivory. *Journal of Ecology* **89**, 578-588.

Moawad, H. A. & Beck, D. P. 1991 Some characteristics of *Rhizobium leguminosarum* isolates from un-inoculated field-grown lentil. *Soil Biology & Biochemistry* **23**, 933-937.

Moawad, H. A., El-Din, S. M. S. B. & Abdel-Aziz, R. A. 1998 Improvement of biological nitrogen fixation in Egyptian winter legumes through better management of *Rhizobium*. *Plant and Soil* **204**, 95-106.

Mueller, U. G., Poulin, J. & Adams, R. M. M. 2004 Symbiont choice in a fungus-growing ant (Attini, Formicidae). *Behavioral Ecology* **15**, 357-364.

Noë, R. & Hammerstein, P. 1994 Biological markets: supply and demand determine the effect of partner choice in cooperation, mutualism and mating. *Behavioral Ecology and Sociobiology* **35**, 1-11.

Noë, R., Vanschaik, C. P. & Vanhooff, J. 1991 The market effect - an explanation for pay-off asymmetries among collaborating animals. *Ethology* **87**, 97-118.

Oba, H., Tawaraya, K. & Wagatsuma, T. 2001 Arbuscular mycorrhizal colonization in *Lupinus* and related genera. *Soil Science and Plant Nutrition* **47**, 685-694.

Quigley, P. E., Cunningham, P. J., Hannah, M., Ward, G. N. & Morgan, T. 1997 Symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* collected from pastures in south-western Victoria. *Australian Journal of Experimental Agriculture* **37**, 623-630.

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

SAS Institute, I. 2004 *SAS/STAT 9.1 User's guide*. Cary, NC: SAS Institute Inc.

Simms, E. L. & Bever, J. D. 1998 Evolutionary dynamics of rhizopine within spatially structured rhizobium populations. *Proceedings of the Royal Society of London Series B Biological Sciences* **265**, 1713-1719.

Simms, E. L. & Taylor, D. L. 2002 Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology* **42**, 369-380.

Singleton, P. W. & Stockinger, K. R. 1983 Compensation against ineffective nodulation in soybean. *Crop Science* **23**, 69-72.

Soberon, J. M. 1985 Cheating and taking advantage in mutualistic interactions. In *The Biology of Mutualism* (ed. D. H. Boucher), pp. 192-216. London: Croom Helm.

Sprent, J. 2003 Plant biology - Mutual sanctions. *Nature* **422**, 672-674.

Sprent, J. I., Sutherland, J. M. & Faria, S. M. d. 1987 Some aspects of the biology of nitrogen-fixing organisms. *Philisophical Transactions of the Royal Society of London, Series B* **317**, 111-129.

Stepkowski, T., Swiderska, A., Miedzinska, K., Czaplinska, M., Swiderski, M., Biesiadka, J. & Legocki, A. B. 2003 Low sequence similarity and gene content of symbiotic clusters of *Bradyrhizobium* sp. WM9 (*Lupinus*) indicate early divergence of "lupin" lineage in the genus *Bradyrhizobium*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **84**, 115-124.

Thrall, P. H., Burdon, J. J. & Woods, M. J. 2000 Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian legumes: Interactions within and between genera. *Journal of Applied Ecology*. **37**, 52-65.

Trinick, M. J. 1977 Vesicular-arbuscular infection and soil phosphorus utilization in *Lupinus* spp. *New Phytologist* **78**, 297-304.

Triplett, E. W. & Sadowsky, M. J. 1992 Genetics of competition for nodulation of legumes. *Annual Review of Microbiology* **46**, 399-428.

van Berkum, P. & Fuhrmann, J. J. 2000 Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. *International Journal of Systematic and Evolutionary Microbiology* **50**, 2165-2172.

Vasquez-Arroyo, J., Sessitsch, A., Martinez, E. & Peña-Cabriales, J. J. 1998 Nitrogen fixation and nodule occupancy by native strains of *Rhizobium* on different cultivars of common bean (*Phaseolus vulgaris* L.). *Plant and Soil* **204**, 147-154.

Wadisirisuk, P. & Weaver, R. W. 1985 Importance of bacteroid number in nodules and effective nodule mass to dinitrogen fixation by cowpeas. *Plant and Soil* **87**, 223-231.

West, S. A., Kiers, E. T., Pen, I. & Denison, R. F. 2002a Sanctions and mutualism stability: when should less beneficial mutualists be tolerated? *Journal of Evolutionary Biology* **15**, 830-837.

West, S. A., Kiers, E. T., Simms, E. L. & Denison, R. F. 2002b Nitrogen fixation and the stability of the legume-rhizobium mutualism. *Proceedings of the Royal Society of London Series B* **269**, 685-694.

Wilkinson, D. M. 1997 The role of seed dispersal in the evolution of mycorrhizae. *Oikos* **78**, 394-396.

Wilson, W. G., Morris, W. F. & Bronstein, J. L. 2003 Coexistence of mutualists and exploiters on spatial landscapes. *Ecological Monographs* **73**, 397-413.

Table 1. Characteristics of *Bradyrhizobium* strains.

strain	wild host*	location	shoot mass (g)**	ranked symbiotic benefit
LA11.1	*L. arboreus*	South Grassland	1.93	low
LA17.1	*L. arboreus*	North Dunes	2.14	medium
LB16.1	*L. bicolor*	Mussel Point	2.36	high

(* Host from which strain was isolated. ** Average aboveground dry mass after 20 weeks of 48 *L. arboreus* plants inoculated with the strain. Strains differed significantly [$F_{2,126} = 12.46$, $P \leq 0.0001$]; experiment-wide 95% confidence limit = 0.12 grams.)

Table 2. Contingency tables of strain identity versus nodule size class after accounting for differences among plants.

a. Strains LB16-1 vs. LA11-1

strain	rank	large	small
LB16-1	best	10	8
LA11-1	worst	8	25

$\chi_1^2 = 5.00$, $p < 0.03$

b. Strains LA17-1 vs. LA11-1

strain	rank	large	med	small
LA17-1	med*	11	12	6
LA11-1	worst	0	18	8

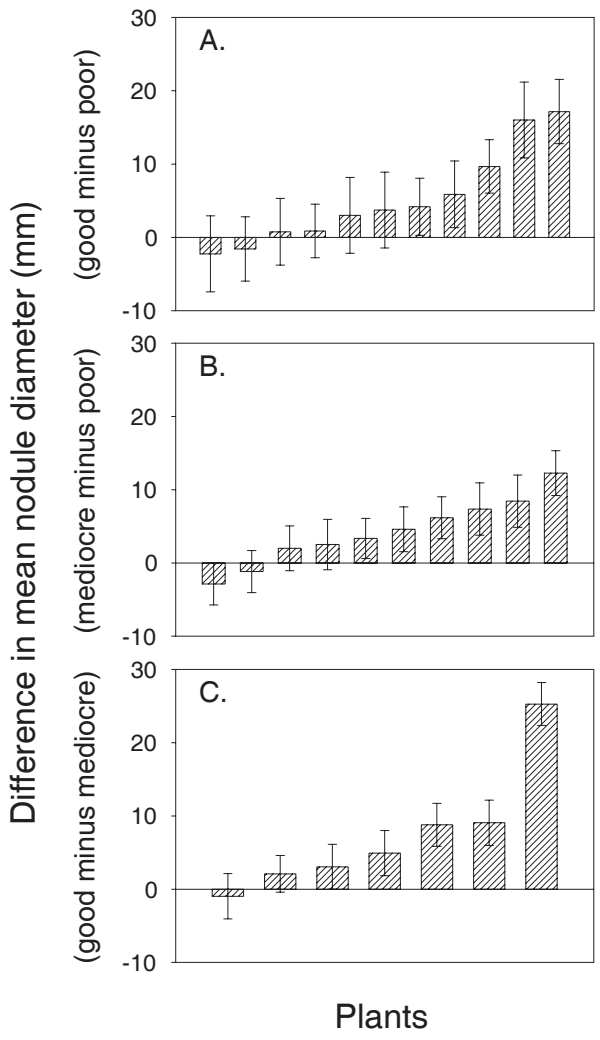
$\chi_1^2 = 12.36$, $p < 0.002$, * med = mediocre

c. Strains LB16-1 vs. LA17-1

strain	rank	large	small
LB16-1	best	6	2
LA17-1	med*	6	17

$\chi_1^2 = 5.99$, $p < 0.02$, * med = mediocre

Figure 1. Within plant average difference in sizes of nodules occupied by best versus worst strain. Plants inoculated by A. strains LB16-1 (good) and LA11-1 (poor), B. strains LA17-1 (mediocre) and LA11-1 (poor), C. strains LB16-1 (good) and LA17-1 (mediocre). Bars indicate 1 S.E.


Difference in mean nodule diameter (mm)
A.
(good minus poor)
B.
(mediocre minus poor)
C.
(good minus mediocre)
30
20
10
0
-10
Plants