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Origins of cheating and loss of symbiosis in wild *Bradyrhizobium*

J. L. SACHS, M. O. EHINGER & E. L. SIMMS

Integrative Biology, University of California, Berkeley, CA, USA

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Abstract

Rhizobial bacteria nodulate legume roots and fix nitrogen in exchange for photosynthates. These symbionts are infectiously acquired from the environment and in such cases selection models predict evolutionary spread of uncooperative mutants. Uncooperative rhizobia – including nonfixing and non-nodulating strains – appear common in agriculture, yet their population biology and origins remain unknown in natural soils. Here, a phylogenetically broad sample of 62 wild-collected rhizobial isolates was experimentally inoculated onto *Lotus strigosus* to assess their nodulation ability and effects on host growth. A cheater strain was discovered that proliferated in host tissue while offering no benefit; its fitness was superior to that of beneficial strains. Phylogenetic reconstruction of *Bradyrhizobium* rDNA and transmissible symbiosis-island loci suggest that the cheater evolved via symbiotic gene transfer. Many strains were also identified that failed to nodulate *L. strigosus*, and it appears that nodulation ability on this host has been recurrently lost in the symbiont population. This is the first study to reveal the adaptive nature of rhizobial cheating and to trace the evolutionary origins of uncooperative rhizobial mutants.

Introduction

Mutualistic bacterial symbionts are widespread in animal and plant hosts, and these bacteria can strikingly alter host phenotype (Sprent *et al.*, 1987; Ruby, 1996; Dethlefsen *et al.*, 2007). Yet the cooperative nature of these symbioses is thought to be evolutionarily unstable, because horizontal transmission hinders strong positive correlation between the fitness interests of bacteria and host (Fine, 1975; Frank, 1996). In particular, two evolutionary processes might challenge the persistence of these mutualistic interactions: abandonment and cheating (Sachs & Simms, 2006). Many conditions might select for symbionts to abandon host interaction and thus return to independent existence. For example, if hosts are rare or poorly matched and provide inadequate benefits (Keeler, 1985; Thompson, 2005), if competitors or pathogens disrupt beneficial interactions (Bronstein, 2003; Wilson *et al.*, 2003), or if alternative resources (outside options) become accessible to the symbionts (Allen, 1991; Paracer & Ahmadjian, 2000). Alternatively, fitness costs of providing benefits to hosts could favour cheater symbionts, which exploit the host for fitness benefits without adequate reciprocation (Wright, 1969; Axelrod & Hamilton, 1981; Soberon & Martinez-Del Rio, 1985; Bronstein, 2001, 2003; Yu, 2001; Sachs *et al.*, 2004). Models for the maintenance of mutualism predict that cheater symbionts are counter-selected under certain conditions, for instance if hosts and symbionts exhibit long-term or repeated interactions (Queller, 1985; Connor, 1995; Sachs *et al.*, 2004; Sachs & Wilcox, 2006; Foster & Wenseleers, 2006) or if hosts exhibit control over symbionts such that they can exclude or sanction uncooperative partners (Bull & Rice, 1991; Frank, 1995; Denison, 2000; Simms & Taylor, 2002; West *et al.*, 2002; Kiers *et al.*, 2003; Sachs *et al.*, 2004; Simms *et al.*, 2006).

Natural selection imposed by hosts during infection is often thought to favour beneficial symbionts (Denison, 2000; Sachs *et al.*, 2004), for instance when hosts plants of rhizobia (Kiers *et al.*, 2003, 2006; Simms *et al.*, 2006) and mycorhizae (Bever *et al.*, 2009) selectively reward beneficial strains or actively sanction harmful ones.

Correspondence: Joel L. Sachs, Department of Biology, University of California, Riverside, CA 92521, USA.
Tel.: +1 951 827 6357; fax: +1 951 827 4286; e-mail: joels@ucr.edu

However, host-driven selection on symbiotic bacteria is limited by the extent to which bacteria replicate in hosts, as opposed to in the environment (Sachs & Simms, 2008). Unlike maternally acquired bacterial symbionts which often exhibit ancient, obligate interactions with host lineages (e.g. Moran *et al.*, 2009), most bacterial partners are facultative and infectiously acquired, including nitrogen-fixing bacteria in plant roots (Sprent *et al.*, 1987), bioluminescent bacteria in invertebrates (Ruby, 1996) and the gut bacterial communities of mammals (Dethlefsen *et al.*, 2007) all of which can potentially retain extensive environmental phases between host association (Savage, 1977; Brockwell *et al.*, 1987; Ruby, 1996; Duodu *et al.*, 2005; Sachs & Simms, 2008). Evolution during free-living phases can favour bacterial traits that promote environmental existence at a cost to symbiotic traits (Sachs & Simms, 2008).

A phylogenetic framework is particularly useful to address the stability and evolutionary breakdown of symbiotic cooperation, because the evolutionary gain and loss of symbiotic traits can be empirically studied. For instance, nonsymbiotic lineages arising from within clades of symbionts suggest the evolution of host abandonment (Sachs & Simms, 2006, 2008). On the other hand, cheating symbionts might arise in two ways: from within a mutualist lineage or from other origins (Bronstein, 2001; Sachs & Simms, 2006). In the former, cheater mutants evolve from cooperative ancestors via an evolutionary reversal (e.g. Pellmyr *et al.*, 1996; Pellmyr & Leebens-Mack, 2000; Machado *et al.*, 2001; Als *et al.*, 2004). In the latter, cheaters emerge from lineages with no evolutionary history of cooperation (Soberon & Martinez-Del Rio, 1985; Bronstein, 2001; Yu, 2001). A fascinating aspect of both mutualistic and pathogenic bacteria is that key host-associated phenotypes are often expressed from mobile plasmids or integrated genome islands (Batut *et al.*, 2004). The gain, loss and transfer of these loci provide mechanisms by which host-associated traits can evolve rapidly (Sullivan *et al.*, 1995).

Rhizobia are bacterial symbionts that infect and form nodules on legume roots and differentiate into specialized endosymbiotic cells called bacteroids, which fix atmospheric nitrogen in exchange for photosynthates (Sprent *et al.*, 1987; Lodwig *et al.*, 2003). Both nodulation and nitrogen fixation traits have evolved via repeated lateral transfer of symbiotic loci (Young & Haukka, 1996; Moulin *et al.*, 2004). Nodulation by effective rhizobia can improve legume growth by 10-fold over infection by ineffective strains (Burdon *et al.*, 1999), and in turn, infecting rhizobia can gain fitness benefits, potentially via release into the soil from senescent nodules (Kiers *et al.*, 2003; Denison & Kiers, 2004a,b; Simms *et al.*, 2006). Nonnodulating rhizobia have been found in agricultural soils and often are closely related to symbiotic strains (Segovia *et al.*, 1991; Sullivan *et al.*, 1996; Pongsilp *et al.*, 2002). But, it is unknown whether these rhizobia ancestrally lack symbiosis loci or whether symbiotic function has been lost in populations (Sachs & Simms, 2006, 2008). Nodulating strains that fail to fix or fix poorly have also been found in agricultural settings (Quigley *et al.*, 1997; Moawad *et al.*, 1998; Denton *et al.*, 2000; Chen *et al.*, 2002; Collins *et al.*, 2002) and in at least one study of unmanaged soils (Burdon *et al.*, 1999). Nonfixing or poorly strains (symbiotically ineffective) are often thought to represent an adaptive uncooperative strategy (Denison, 2000; Denison & Kiers, 2004a) in which the rhizobia redirect plant carbon towards selfish ends (Hahn & Studer, 1986; Lopez *et al.*, 1995; Denison, 2000; Denison & Kiers, 2004a,b) instead of channelling it into the energetically expensive nitrogen fixation pathway (Trainer & Charles, 2006). But in contrast to these predictions, little evidence is available to suggest that nonfixing strains obtain greater fitness than do fixing strains. In some cases, host sanctions can constrain uncooperative rhizobia. For example, soybeans (*Glycine max*) and lupines (*Lupinus arboreus*) can preferentially allocate resources to nodules that house more effective strains (Singleton & Stockinger, 1983; Singleton & Van Kessel, 1987) and cause nodules with symbiotically ineffective (nonbeneficial) rhizobia to be smaller and bear fewer bacteria (Kiers *et al.*, 2003, 2006; Simms *et al.*, 2006). Yet there are studies of soybeans (Marco *et al.*, 2009) and other legume species that did not uncover evidence consistent with sanctions (Atkins *et al.*, 1984; Pate *et al.*, 1984), and research on wild rhizobia (Abdalla, 1992) and lab-generated mutants (Lodwig *et al.*, 2003) has discovered symbiotically ineffective rhizobia that attained higher nodule mass than beneficial strains.

We examined the symbiotic quality of *Bradyrhizobium* in the context of a phylogenetic hypothesis to investigate the evolution and origins of uncooperative rhizobia. We conducted an initial screen (IS) of the symbiotic quality of 62 diverse rhizobial isolates and then used a comparative inoculation (CI) study of a phylogenetically informed sample of rhizobial isolates to simultaneously measure host and bacterial fitness. Three main questions were investigated: (i) Do uncooperative rhizobia, including symbiotically ineffective (nonbeneficial) and nonnodulating strains, exist in natural populations, (ii) Can symbiotically ineffective rhizobia be favoured by natural selection, in the sense that they have superior fitness compared to beneficial strains, and (iii) Do uncooperative rhizobia, including symbiotically ineffective and nonnodulating strains, evolve from mutualist ancestors or do they represent independently evolved lineages?

Materials and methods

Collection of wild *Bradyrhizobia*

Bradyrhizobium sp. were isolated from *Lotus heermannii*, *Lotus micranthus*, *Lotus strigosus and Lotus wrangelianus* at Bodega Marine Reserve and Sonoma Coast State Park as described in Sachs *et al.* (2009). Briefly, intact plants were excavated from the soil, the root systems washed in

tap water, and nodules were dissected from roots, surface sterilized, rinsed, crushed and streaked onto two replica plates of solid arabinose-gluconate media. Root sections of ~1 cm length were also dissected from each plant, incubated individually in a sterile solution of 0.01% Tween 20 (Fisher Scientific, Fair Lawn, NJ, USA) and vortexed to remove root-surface bacteria, which include rhizobia that nodulate *Lotus* as well as those that do not (Sachs *et al.*, 2009). The resulting solution was serially diluted and plated on a glucose-based rhizobium defined medium (G/RDM; Sullivan *et al.*, 1996), and colonies matching the phenotypes of those cultured from host nodules were selectively isolated and screened on G/RDM agar medium, yeast-mannitol agar medium (YM; Somasegaran & Hoben, 1994) and Luria-Bertani medium (LB; Somasegaran & Hoben, 1994).

Using selected taxa from a previous phylogeny of the *Its* (Internal transcribed spacer between the 16s and 23s ribosomal subunits; Sachs *et al.*, 2009), a representative subset of 62 isolates (H_0: clades did not differ in sampling frequency, χ^2_1, $P > 0.2$) was chosen for additional genotyping and phenotyping (an 'initial screen' of nodulation ability and growth effects on hosts). We concentrated mainly on isolates from *L. strigosus* hosts, the species that we used for inoculation assays. The 62 isolates were sampled from *L. strigosus* (37 root-surface samples, 12 nodule samples, respectively), *L. heermannii* (5, 1), *L. micranthus* (3, 2) and *L. wrangelianus* (one root surface). One *Methylobacterium* sp. clone from a *L. strigosus* root-surface sample was tested, because members of this genus sometimes nodulate legumes (Sawada *et al.*, 2003). All *Its* sequences came from previous work (Sachs *et al.*, 2009), and for the present study we additionally PCR amplified DNA at portions of the nitrogenase α-subunit locus (*nifD*, 831nt aligned) (Parker, 2000), the dinitrogenase reductase locus (*nifH*, 672nt aligned) (Vinuesa *et al.*, 2005) and an intergenic region between nodulation loci (*nodD-A* spacer, 1173nt aligned), all located on the presumed symbiosis island (Kaneko *et al.*, 2002), for a total of 3986 sequenced nucleotides (2676 on the symbiosis island + 1310 from the *Its*). We used previously published protocols for *nifD* (Primers P11, P12; Parker, 2000) and *nifH* (Primers 40F, 817R;Vinuesa *et al.*, 2005). The *nodD-A* spacer primers (Forward primer: GTGTTCTATCGAAACAATCG; Reverse primer; ATNCCRAGCCCCTCNAGATC) amplify a region of 1173 nt corresponding to the coordinates 9669 bp and 10842 bp of the *Bradyrhizobium* sp. WM9 nodulation region 1 (GenBank: AF222753.1). The amplification product consists of a noncoding region, a hypothetical 15.0 KDa protein similar to a styrene-response regulator and the 5′ end of the *NodA* gene. The PCR protocol for this reaction was 95 °C for 2 min, 34 cycles of denaturation (15 s, 92 °C), annealing (55 °C for 30 s) and extension (90 s, 72 °C), followed by a final extension (5 min, 72 °C). PCR fragments were sequenced using an Applied Biosystems 96 capillary 3730xl DNA Analyzer (Foster City, CA, USA) at UC sequencing facilities (Berkeley, Riverside, CA, USA). Only unambiguous sequences in which single nucleotide peaks could be resolved for each DNA base were included in the study. All cultured colonies were successfully sequenced and verified on GenBank (NCBI, BLAST; Altschul *et al.*, 1997).

Phylogenetic reconstruction and analysis

For the *Its* tree, we used a pruned version of a phylogenetic reconstruction from a previous analysis (Sachs *et al.*, 2009) in which only 61 of the inoculated isolates and one outgroup taxon were included (the *Methylobacterium* genotype was not included in the phylogenetic analyses because it was greatly divergent from the other strains). We used the *Its* from the fully sequenced *B. japonicum* USDA110 strain as the outgroup (Accession #BA000040.2). Sequences were aligned using Clustal-W (Thompson *et al.*, 1994) with default parameters. For the reconstructions of *Its*, *nif*D, and *nifH*, we also used homologous regions from *B. japonicum* USDA110 as outgroups. We could not find a homolog for the *nodD-A* spacer in this genome, so these trees were left unrooted. Gaps were treated as missing data and only unambiguously aligned positions were used to construct phylogenetic hypotheses. Model fitting was performed with Mr. Modeltest 2.2 (Nylander *et al.*, 2004), and best-fit models were identified using the Akaike Information Criterion (Akaike, 1973). Best-fit models for the *Its* were identified in a previous analysis (Sachs *et al.*, 2009). For *nifD* and *nifH*, the best-fit model was generalized time reversible (GTR) + G; for the *nodD-A* spacer, the best-fit model was the GTR + I. All phylogenetic trees were reconstructed using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) with the following settings: eight simultaneous chains, 10^6 generations, a heating temperature of 0.02, a 'burnin' of 9001 trees and two parallel runs starting with random trees. Using a sample of the post-'burnin' trees, a majority rule consensus tree was reconstructed using the 'sumt' command on MrBayes. A plot of log-likelihood scores of sampling points (sample frequency = 100) against generation number was observed in each case to ensure that stationarity had been reached during the 'burnin' period.

Host growth effect and nodulation ability on *L. strigosus* were mapped on the *Its* tree, and ancestral state reconstruction was performed using parsimony (Maddison & Maddison, 1992) and Bayesian stochastic character mapping (Huelsenbeck *et al.*, 2003; SIMMMAP; Bollback, 2006). Evidence for horizontal transfer of symbiosis loci was examined by assessing congruence among gene genealogies using cophylogeny randomization tests in TREEMAP 1.0 & 2.0 (Page, 1994; Page & Charleston, 1998). This procedure estimates the number of divergence events shared by two phylogenies using a heuristic search, then generates a set of 1000 random topologies for one of the trees and tests whether the empirically

estimated number of co-divergence events deviates significantly from the number of co-divergence events in the randomly generated dataset. Congruence among trees was also assessed using the maximum χ^2 test of recombination (Maynard Smith, 1992; Piganeau *et al.*, 2004).

Inoculation tests

A detailed description of our inoculation protocol can be found in Sachs *et al.* (2009). *Lotus strigosus* was used for inoculation assays because it is a permissive host; we have found it to form nodules with bradyrhizobia collected from the nodules of all other local *Lotus* spp. (Sachs *et al.*, 2009). *Lotus strigosus* fruits were collected at Bodega Marine Reserve, CA, USA. Seeds were surface sterilized in bleach, rinsed in sterile ddH_2O, nick scarified and germinated in sterile ddH_2O. Seedlings were planted into bleach-sterilized pots filled with autoclaved quartzite sand and incubated in a growth chamber (20 °C, 80% relative humidity, 12 : 12 day/night cycle, 2× daily misting, 14 days) before being transferred to a greenhouse under ~50% shade for hardening (14 days, 2× daily misting). Once in the greenhouse, plants were fertilized weekly with Jensens's nitrogen-free solution (Somasegaran & Hoben, 1994), beginning with 2 mL per seedling, increasing by 1 mL each week until reaching 5 mL per plant, which was used thereafter. *Bradyrhizobium* cultures were initiated from ~2 μL of original frozen stock inoculated into 50 mL of liquid modified arabinose gluconate (MAG) media and incubated to logarithmic phase growth (29 °C, 180 rpm, 72 h). Bacterial concentrations were estimated via light absorbance. Grown cultures were lightly centrifuged (4000 rpm, 20 min.) and re-suspended in prewarmed sterile ddH_2O to concentrations of 10^8 cells mL^{-1}. Treatment plants were inoculated with 5 mL of re-suspended cultures, and control plants were inoculated with 5 mL of sterile ddH_2O.

Initial screen

Greenhouse assays of isolates were conducted from November 2005 to June 2006 to test (i) their ability to nodulate *L. strigosus* (reported in Sachs *et al.*, 2009) and (ii) their effect on *L. strigosus* growth. Each isolate was tested on a group of 10 seedlings, including five inoculated and five uninoculated controls, which were paired by size (via a leaf count). To avoid cross-contamination during inoculation, plants were grouped by rhizobial strain. Plants were harvested 8 weeks after inoculation, roots inspected under the dissecting scope for nodules, and host plant fitness estimated from oven-dried aboveground biomass. We measure biomass at 8 weeks, because this is the period within which nodules form in the greenhouse and subsequently nodule senescence begins. We conducted a small experiment to correlate leaf count at 8 weeks after inoculation (a nondestructive estimate of biomass) and seed-set a month later for *L. strigosus* plants in the greenhouse. We have found a positive, but nonsignificant relationship ($n = 26$, $R = 0.10$, $P = 0.45$) between leaf count and seed number. Rhizobial strains were classified as non-nodulating on *L. strigosus* if no nodules were found in any of the five inoculated plants. Rhizobial strains were classified as symbiotically ineffective if the five inoculated plants exhibited nodules, but did not grow significantly more than the five paired uninoculated control plants. No evidence of nodulation was found in any of the control plants.

Comparative inoculation test (CI)

Results from the IS were used to select 14 strains representing contrasting phenotypes from multiple clades, including nodulating and non-nodulating as well as beneficial and symbiotically ineffective strains (as tested on *L. strigosus*). Axenic seedlings were prepared as described earlier and sorted by size into six spatial blocks. Within each block, two pairs of seedlings were assigned at random to each *Bradyrhizobium* strain. Within each pair, one seedling was randomly chosen to be the uninoculated control; the other seedling was inoculated with the appropriate strain. Thus, each of 14 rhizobial strains was inoculated onto 12 plants, each plant matched with an uninoculated control, for a total of 336 plants. Plants were spaced at least 10 cm apart and mist-watered to avoid cross-contamination. All plants were harvested 8 weeks after inoculation, except as described later. Plants were un-potted, separated into roots and shoots, and shoots were oven-dried and weighed to measure above-ground growth rate, a component of host plant fitness. All plants were examined for evidence of nodulation, which was not found in any controls.

Because symbiotic rhizobia reproduce and are ultimately released from *Lotus* nodules into the soil (Mergaert *et al.*, 2006), we measured nodule number and mass for all nodulated plants, which are both commonly measured components of rhizobial fitness (Denison, 2000; Muller *et al.*, 2001; Simms & Taylor, 2002; Denison & Kiers, 2004a). However, culturing from nodules provides a more direct estimate of the number of reproductively viable rhizobia within each nodule. We therefore dissected six randomly chosen nodules of varying size from a random subset of 24 plants in blocks three and six. Two replicate plants from each of the six nodulating strains were sampled during each of the sixth- and eighth-week harvests. For each nodule, we obtained wet mass, and then surface sterilized, rinsed and crushed the nodule with a sterile pestle and plated nodule bacteria on two MAG plates each using dilutions of 10^{-2} and 10^{-4}. Mean population per nodule was calculated using the mean colony count of at least two plates; only plates exhibiting between 20 and 700 colonies were counted. All nodules remaining on the plant were dissected and oven-dried to obtain dry mass. A subset of these nodules was weighed both wet and dry to obtain

a wet–dry mass curve (Dry = 13.37565 + 0.130628 Wet, $r^2 = 0.967$, $P < 0.001$, $n = 36$). Total rhizobial fitness on a plant was calculated by multiplying the mean bacterial population per nodule times the number of nodules, as well as by multiplying mean bacterial population per dry nodule mass times the total nodule dry mass of the plant. Both methods gave similar results and we report only the latter.

Relative plant growth was calculated as the difference in shoot dry mass between the inoculated and uninoculated plant within each pair, divided by the dry mass of the uninoculated control plant. This dependent variable was first analysed with a split-plot nested analysis of variance (ANOVA), with strain nested within nodulation class (nodulating or non-nodulating on *L. strigosus*) and pair nested within block. The main effect of nodulation class was tested with an F-statistic calculated from the ratio of the nodulation class mean square over the mean square because of strain within nodulation class. The main effect of strain was then tested using split-plot ANOVAS run separately for each nodulation class. Within the nodulating class, planned contrasts were used to compare the average effects of strains from symbiotic Clade 2 with those of strains from other clades. Also within the nodulating class, mixed model ANOVA was used to analyse the main effect of strain on three rhizobial fitness components: (i) total nodule dry mass per plant, (ii) total nodule number per plant and (iii) total bacterial cell number per plant. As with the plant fitness component, planned contrasts were used to compare the average effects of strains from symbiotic Clade 2 with those of strains from other clades. In all cases, block and pair were treated as random effects and strain and nodulation class as fixed effects; the ANOVAS were performed with the MIXED procedure of SAS® 9.2 for the XP Pro platform (SAS Institute, Inc., Cary, NC, USA). The possibility of fitness trade-offs between host and bacteria, across bacterial strains, was tested by calculating pairwise strain mean correlations of rhizobial fitness components (nodule number/host, nodule dry mass/host and total rhizobial population/host) with plant relative growth. Correlations were calculated using JMP® Version 7.0 (SAS Institute, Inc., 1989–2007).

Results

Phylogenetic reconstruction

For the *Its* tree pruned from the previous phylogeny (Sachs *et al.*, 2009; Fig. 1), five well-resolved clades (posterior probabilities ≥ 0.85) incorporate most of the sequenced isolates. Genotypes that nodulated or failed to nodulate *L. strigosus* were most often diverged from each other: 33 of the 36 nodulating isolates clustered into two clades (Clades #1 and #2) that also included a minority of isolates that did not nodulate *L. strigosus*. For consistency, clade designations follow the previous study (Sachs *et al.*, 2009) and not all clades from that study are not represented among the 61 test strains. For the symbiosis loci sequenced here (*nifD*, *nifH*, *nodD-A* spacer) we found that were unable to PCR amplify any of these loci from the isolates that failed to nodulate *L. strigosus* in the inoculation assays (Fig. 1). For *nifD* and the *nodD-A* spacer, we also found the converse: we could successfully PCR amplify these loci in all nodulating isolates. For *nifH*, there were five nodulating isolates that we could not successfully PCR amplify. All negative PCR results were triple checked with positive controls (same PCR mix plus *Its* primers) to confirm the lack of amplification. Among the symbiosis-island loci, *nifD*, *nifH*, *nodD-A* spacer, 15, 15 and 13 genotypes, respectively, were resolved among the sequenced strains (Fig. 2).

IS results

Rhizobial isolates either caused nodules in all the inoculated plants or failed to form nodules in any of them. All 16 nodule isolates successfully nodulated *L. strigosus* (2 isolates from *L. heermannii*, 2 *L. micranthus*, 12 *L. strigosus*; Sachs *et al.*, 2009). Twenty of 46 root-surface isolates also successfully nodulated *L. strigosus* (3 isolates from *L. heermannii*, 17 *L. strigosus*; Sachs *et al.*, 2009). Of the 36 nodulating inocula, 33 strains significantly increased host growth and three caused no detectable effect (strains 2, 14, 38; two-tailed *t*-tests, A = 0.05, $n = 10$). Two of the strains that caused no host growth were isolated from *L. strigosus* (2, 38) and the other was from *L. micranthus* (14). Of the 26 strains that failed to nodulate *L. strigosus*, 22 of the strains caused no detectable growth effects on the hosts (3 isolates from *L. heermannii*, 2 *L. micranthus*, 20 *L. strigosus*, 1 *L. wrangelianus*), three appeared to increase host growth (strains 44, 61 *L. strigosus*; 53, *L. micranthus*) and one appeared to decrease host growth (strain 40, *L. strigosus*).

CI results

Relative *Lotus* shoot biomass (growth) and fitness components of infecting *Bradyrhizobium* strains were measured at either 6 or 8 weeks after inoculation. Both shoot biomass and rhizobial fitness components increased over the 2 weeks between measurements, but results were similar at each time point. Below, we report data from the 8-week time point unless otherwise noted.

Rhizobial effects on host growth

On average, nodulating strains increased plant relative growth more than the strains that failed to nodulate *L. strigosus* (Table 1, $F_{1,12} = 16.46$, $t > 0.005$). Seven of the 14 strains tested in the CI were ones that failed to nodulate *L. strigosus* in the IS (strain numbers 15, 20, 40, 44 47, 48, 53 and 62). Four of these strains were chosen because they appeared to either benefit (44, 47, 53) or harm (40) hosts in the IS. In the CI, we detected no

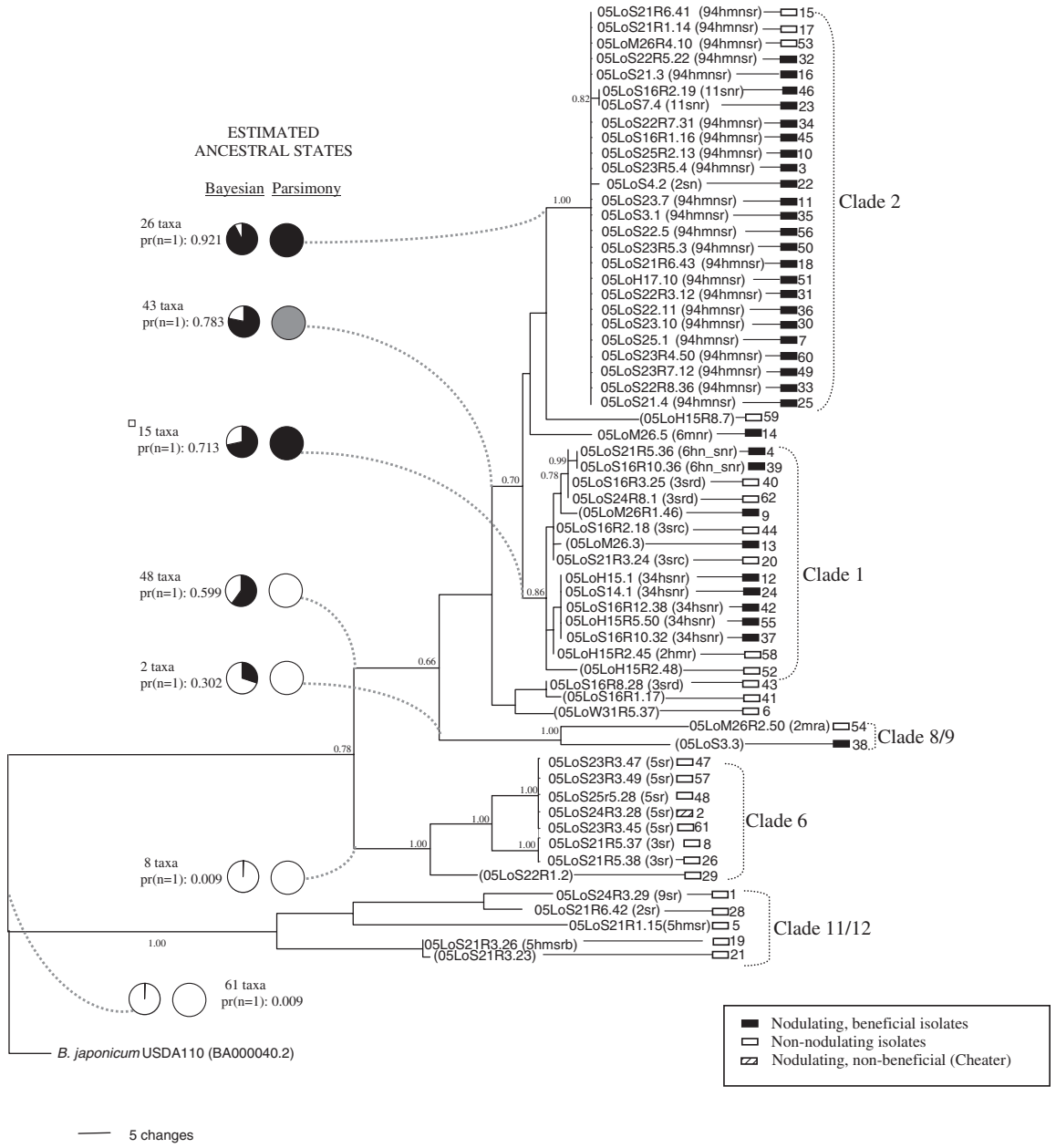

Fig. 1 Bayesian phylogram inferred with the *Its* sequences (1279 nt) of 61 *Bradyrhizobium japonicum* isolates from BMR (from Sachs *et al.*, 2009) and rooted with the *Its* region of USDA strain 110 (GenBank Accession BA000040), for which the whole genome is sequenced. Beginning from the left, taxon labels for rhizobial isolates consist of the year of isolation (05 = 2005), host species (LoA = *Lotus angustissimus*, LoM = *Lotus micranthus*, LoH = *Lotus heermannii*, LoS = *Lotus strigosus*), plant number and nodule or root-surface number (the latter with R followed by root and isolate number). GenBank accession codes (from a previous analysis; Sachs *et al.*, 2009) are listed next in parentheses and consist of the number of times the genotype was originally recovered (in Sachs *et al.*, 2009) followed by the host species (A, M, H, S; see above) and isolate types (N = nodule, R = root surface) from which the genotype was recovered. For unique isolates, the taxon label is used as the GenBank accession and is in parentheses. Symbiotic phenotypes on *L. strigosus* from the initial screen (IS) and comparative inoculation are indicated on the tips of the tree with rectangular labels (Black = nodulated plants grew significantly more than uninoculated controls in IS, White = none of the inoculated plants formed nodules, Striped = nodulated plants did not grow significantly more than controls). Strain numbers (1-62) are given on the right of the symbiotic phenotype labels. Bayesian clade support values (posterior probabilities) are reported for all well-supported clades (pp ≥ 0.70), and clades with support values ≤ 0.50 are collapsed. Ancestral states were estimated at key internal nodes for the binary character of nodulating (black) or non-nodulating on *L. strigosus* (white) using parsimony and Bayesian stochastic character mapping (with an uninformative prior). Bayesian posterior probabilities of the ancestral states (posterior probability of the ancestor being nodulating) are reported using pie charts.

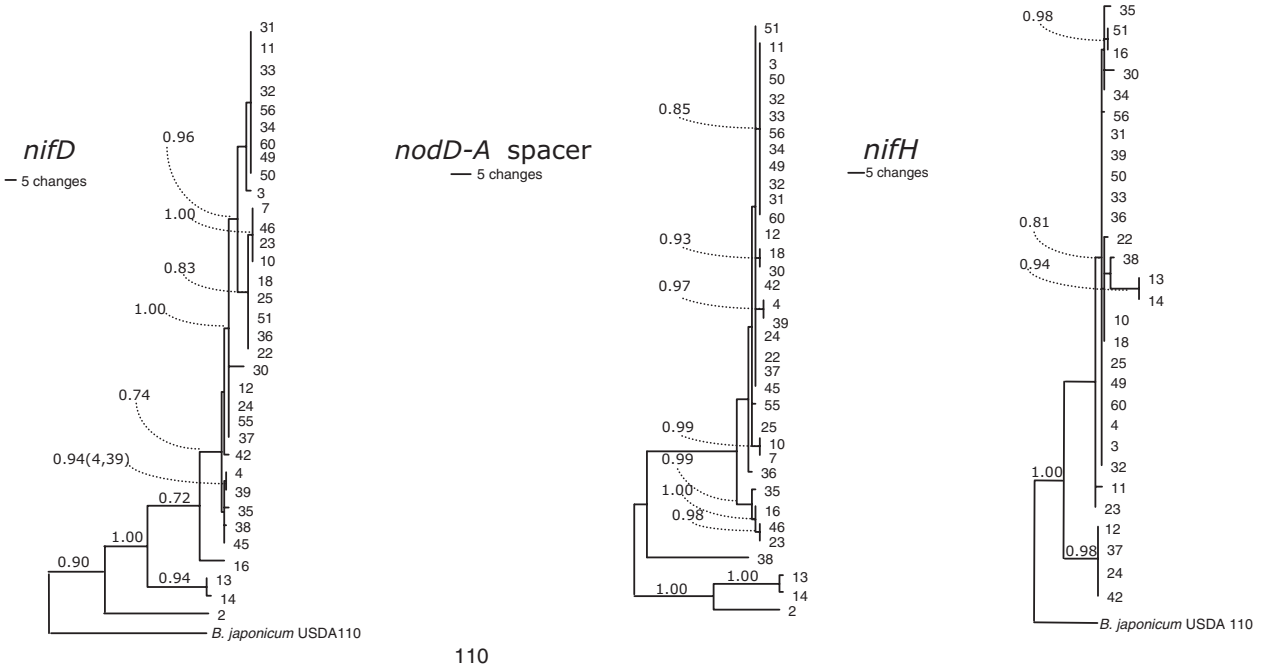

Fig. 2 Bayesian phylograms inferred with *NifD* (a), the *nodD-A* spacer (b), and *NifH* (c). Only nodulating isolates (labelled with strain number) are included in these phylogenies because no symbiosis locus amplified for any of the non-nodulating isolates. Both the *NifD* and *NifH* are rooted with homologous sequences from USDA strain 110 (Accession #BA000040), but no suitable outgroup could be found for the *nodD-A* spacer, so the tree was left unrooted. Bayesian clade support values (posterior probabilities) are reported for all well-supported clades (pp ≥ 0.70).

Table 1 Means and standard errors of inocula phenotypes measured in the comparative inoculation (*$P < 0.0001$)

Strain	Sym.	Rel. growth	Nodules	Nodule mass (mg)	Rhiz. density (mg^{-1})	Rhiz. pop/plant	Rhiz. pop/nod
15	N	0.85 ± 0.06	0	–	–	–	–
20	N	1.31 ± 0.10	0	–	–	–	–
40	N	1.27 ± 0.31	0	–	–	–	–
47	N	1.45 ± 0.20	0	–	–	–	–
48	N	0.84 ± 0.08	0	–	–	–	–
53	N	1.22 ± 0.27	0	–	–	–	–
62	N	1.01 ± 0.11	0	–	–	–	–
2	Y	0.94 ± 0.07	46.25 ± 4.03	2.24 ± 0.1	$1.04 \pm 0.11 \times 10^8$	$2.95 \pm 1.17 \times 10^8$	$6.60 \pm 0.80 \times 10^6$
14	Y	2.31 ± 0.27*	27.0 ± 2.1	6.18 ± 0.64	$1.38 \pm 0.64 \times 10^7$	$2.08 \pm 0.95 \times 10^7$	$1.51 \pm 0.67 \times 10^6$
18	Y	5.99 ± 0.45*	18.5 ± 2.3	7.60 ± 1.07	$1.41 \pm 0.95 \times 10^7$	$4.99 \pm 4.78 \times 10^6$	$4.21 \pm 3.96 \times 10^5$
24	Y	3.90 ± 0.74*	19.5 ± 3.8	7.07 ± 1.28	$1.39 \pm 0.74 \times 10^7$	$1.17 \pm 0.71 \times 10^7$	$1.02 \pm 0.56 \times 10^6$
38	Y	3.79 ± 0.75*	26.6 ± 4.5	6.62 ± 1.23	$2.81 \pm 2.02 \times 10^6$	$1.24 \pm 1.00 \times 10^6$	$1.94 \pm 1.70 \times 10^5$
49	Y	5.01 ± 0.53*	21.6 ± 2.6	7.56 ± 0.96	$3.65 \pm 1.77 \times 10^6$	$8.1 \pm 4.33 \times 10^6$	$1.20 \pm 0.63 \times 10^6$

Sym, strain nodulation status; Y, successful nodulation in all five test plants; N, no nodules detected in any of the test plants upon inspections with a dissecting microscope; Rel. growth, relative growth, calculated as the difference in shoot dry mass (in mg) between the inoculated and uninoculated plant within each pair, divided by the dry mass of the uninoculated control plant; Nods, mean number of nodules; Nodule mass, mean dry mass of nodules per plant, Rhiz. density, mean number of bacteria cultured per mg of dry tissue mass; Rhiz. pop/plant, mean number of bacteria cultured per plant by extrapolation of Rhiz. density; Rhiz. pop/nod, mean number of bacteria cultured per nodule.

significant effects on plant relative growth in strains that failed to nodulate *L. strigosus* (Table 1, $F_{6,41} = 1.64$, $P > 0.16$). Although strain 44 did not form any nodules in the IS, it nodulated all 12 test plants in the CI. This strain is ambiguous, and we suspect was contaminated or switched with another strain in the CI, because the archived culture subsequently showed no signs of nodulation and we were unable to PCR amplify any symbiosis loci from it. Thus, strain data from strain 44 were removed from the CI. Five of the nodulating strains

Table 2 *A priori* contrasts among clades in growth effects on plants

Clade contrasts	Relative plant growth	$F_{1,46}$	P
18 & 49 vs. 24	2.72 ± 1.026	7.04	0.04
18 & 49 vs. 14	3.18 ± 0.63	25.67	0.0004
18 & 49 vs. 38	1.71 ± 0.63	7.42	0.04
18 & 49 vs. 2	4.55 ± 0.63	52.56	0.0004

Clade contrasts, pairs of taxa that are compared; Relative plant growth, milligrammes of dry biomass ± one standard deviation.

(14, 18, 24, 38 and 49) caused inoculated hosts to grow significantly more than controls (Table 1). Strain 2 nodulated hosts but provided no growth benefit. Mean dry mass of hosts inoculated with strain 2 was 0.94× that of controls, but this value was not statistically significantly different from 1.00 ($P > 0.8$). Nodulating strains from Clade 2 (18, 49) increased relative host shoot mass significantly more than did nodulating strains of any other clade (Table 2).

Rhizobial fitness on hosts

Nodulating strains varied significantly in both mean total nodule mass (Table 1, $F_{6,18} = 4.62$, $P \leq 0.0009$) and mean nodule number ($F_{6,49} = 10.63$, $P \leq 0.0001$) per plant. *A priori* contrasts showed that both patterns were driven entirely by strain 2, which produced significantly more nodules (7.37 ± 0.45, $F_{1,48} = 22.23$, $P \leq 0.0001$) and yielded significantly less total nodule mass ($F_{1,49} = 52.81$, $P \leq 0.0001$) than did other nodulating strains. The total rhizobial population size estimated per plant differed significantly among strains ($F_{5,18} = 5.88$, $P \leq 0.002$, Table 1), and this effect was also because of the large difference ($F_{1,18} = 29.32$, $P \leq 0.0001$) between strain 2 ($2.95 \pm 1.17 \times 10^8$) and the others ($9.36 \pm 2.85 \times 10^6$). There was no significant phenotypic correlation between total nodule mass and rhizobial population in the sixth-week harvest ($r = -0.40$, $P > 0.2$, $n = 12$) and a weak negative correlation between total nodule mass and rhizobial population in the eighth-week harvest data ($r = -0.76$, $P < 0.004$, $n = 12$). Strain 2 drove this latter pattern, as it induced the smallest nodules but grew profusely within those nodules. Strain 2 reached population densities greater than 10^8 cells mg^{-1} dry nodule mass, which is fivefold greater than the next highest genotype, and more than nine-fold greater than the mean for all other strains.

Correlations between Lotus and Bradyrhizobium fitness

For each of the nodulating strains, we examined phenotypic correlations between the effect of inoculation on plant shoot mass (inoculated minus paired control) and the number and total mass of nodules. All strains except 2 generated significant positive phenotypic correlations between nodule mass/plant and effect on host shoot mass (Table 3). We found a similar pattern for nodule number except that correlations were weaker (Table 3).

Table 3 Within strain, among plant pairwise phenotypic correlations of shoot mass with nodule number and total nodule mass for the comparative inoculation ($n = 12$)

Strain	18	49	24	14	38	2
Nodule number	0.66*	0.84**	0.87***	0.72*	0.67*	−0.21
Nodule mass	0.93****	0.93***	0.92****	0.71*	0.98****	0.14

****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.1$.

Across strains, the mean effect of inoculation on plant shoot mass was positively correlated with mean total nodule mass ($r = 0.87$, $P \leq 0.01$), negatively correlated with mean total nodule number ($r = -0.92$, $P \leq 0.003$) and weakly negatively correlated with mean total rhizobial population size/plant ($r = -0.78$, $P \leq 0.07$). However, jackknifing identified strain 2 as an outlier. When this strain was removed from the analysis, only the correlation with nodule number remained significant ($r = -0.87$, $P \leq 0.03$).

Ancestral character mapping

The ability to nodulate *L. strigosus* appears to have been gained and lost in the *Its* phylogeny, as indicated by maximum parsimony and Bayesian ancestral character mapping (Fig. 1). The inferred ancestral condition at the base of the tree is non-nodulating on *L. strigosus* (posterior probability of nodulating, pn < 0.01; Bayesian clade support; pp = 1.00). The ability to nodulate *L. strigosus* has been gained at least twice, once in the cheater (strain #2) in Clade 6 and at least once leading to Clades 1 (pn = 0.71, pp = 0.86) and 2 (pn = 0.92, pp = 1.00). Strain #2 is nested in Clade 6, the members of which otherwise do not nodulate *L. strigosus* (pn = 0.01, pp = 1.00). This observation is consistent with its recent acquisition of *L. strigosus* nodulation via horizontal gene transfer, because the sequence of the *Its* region in the cheater is identical to that of four isolates that failed to nodulate *L. strigosus*. Being conservative, the ability to nodulate *L. strigosus* has been lost between one and five times. Clade 2 exhibits at least one unambiguous loss from an inferred nodulating ancestor (strains 15, 17, 53). Clade 1 exhibits between two and four independent lineages that cannot nodulate *L strigosus*, but the ancestral condition of this clade is more ambiguous.

Congruence among loci

Randomization tests using TREEMAP (Page, 1994; Page & Charleston, 1998) revealed that the overall *Its* phylogeny shares a significant number of co-divergence events with each symbiosis-island-locus phylogeny (e.g. more than expected purely by chance; Table 4). Hence, horizontal transmission events of symbiosis loci are not so common as to erase a shared history with the rest of the genome. We created a combined symbiosis-locus tree

Table 4 Tests of congruence, recombination and phylogenetic co-diversification among loci

Loci	*Its*	*NifD*	*NifH*
NifD	< 0.001*/15†/10.41‡	–	–
NifH	< 0.001*/10†/6.98‡	0.430*	–
NodD_A	< 0.001*/19†/11.86‡	0.004*	0.023*

For all pairwise comparisons of loci we report: *the *P* values of a maximum chi-square recombination tests (Maynard Smith, 1992). For all pairwise comparisons between the *Its* region and symbiosis-island loci, we also report: †the estimated number of co-diversification events among phylogenies via a heuristic search on Treemap 2.0 (Charleston, 1998).
‡Random expectation of the number of co-diversification events among phylogenies via 100 randomizations of the *Its* phylogeny compared to the phylogenies of symbiosis-island loci.

(*NifD* + *nodD-A* spacer) to map co-divergence events between the *Its* locus and the symbiosis island (the *nifH* locus was not included in this analysis because five of the nodulating strains could not be amplified). Co-divergence mapping of the phylogenies using TREEMAP (Page, 1994; Page & Charleston, 1998) shows that the *Its* tree shares similar topology with a combined *NifD*, *nodD-A* spacer tree (Fig. 3). Nonetheless, the phylogeny of each symbiosis locus exhibits multiple well-supported clades (pp ≥ 0.70) that are in conflict with well-supported clades on the *Its* phylogeny (Figs 2 and 3) and pairwise comparisons of the symbiosis loci with the *Its* detected significant incongruence using recombination detection methods (Table 4), suggesting that horizontal symbiosis-locus transfer events have occurred among *Its* lineages. We attempted Jungles analyses using Treemap 2 (Charleston, 1998) to resolve individual symbiosis-transfer events of symbiosis loci, but the conflict among trees was too great to resolve single events.

Discussion

Mutualistic symbioses are ubiquitous, yet these interactions are predicted to be evolutionary unstable except under restrictive conditions, for instance when host and symbiont fitness are correlated by vertical transmission or if hosts exert control over symbiont cooperation (Bull & Rice, 1991; Frank, 1995; Queller, 1985; Connor, 1995; Sachs *et al.*, 2004; Foster & Wenseleers, 2006). If these conditions are not met then uncooperative mutants can invade and the interaction may dissolve (Sachs & Simms, 2006, 2008). The dissolution of a symbiosis is especially likely if symbiotic traits are costly to express and if fitness returns from the interaction are outweighed by the potential benefits of exploiting a partner or abandoning the interaction (Sachs & Simms, 2006, 2008). Uncooperative mutants might be selected to invade in any case in which symbionts deliver costly nutrients or services, for instance breakdown of macromolecules by gut-floral bacteria (Dethlefsen *et al.*, 2007), bacterial bioluminescence (Ruby, 1996) or the bacterial fixation of atmospheric nitrogen. In rhizobial symbioses in particular, the direct carbon costs of nitrogen fixation are known to be very high (Tjepkema & Winship, 1980), but the potential fitness benefits of exploiting or abandoning hosts have remained unclear. The current study is novel in that we (i) study variation in symbiotic quality at an intrapopulation rather than a geographic scale, (ii) compare fitnesses among strains within a population of symbionts that vary in the benefit they provide to hosts and, (iii) use phylogenies to infer the evolutionary origins of uncooperative symbionts.

As predicted by theory (Soberon & Martinez-Del Rio, 1985; Bronstein, 2001, 2003; Yu, 2001; Sachs *et al.*, 2004), the natural population under study harboured uncooperative rhizobia, including mutants that were symbiotically ineffective and non-nodulating on the tested host. The host benefit provided by nodulating strains ranged from large to nonexistent, as has been discovered in other populations (Burdon *et al.*, 1999). Of the 36 nodulating strains tested, only one appeared to be symbiotically ineffective (providing zero benefit), and none of the strains reduced host growth. Nodulation is often assumed to entail a cost to the host, because legume hosts respire more when nodulated (Ryle *et al.*, 1979), and because hypernodulating plant mutants grow poorly (Suzuki *et al.*, 2008), yet we failed to detect such a cost. However, the assay of rhizobial effect on host growth was biased towards detecting positive effects, because hosts were given zero nitrogen and uninoculated controls grew very little. Plants also grew for a relatively short period; longer exposure to symbiotically ineffective strains might reveal a fitness cost of ineffective nodulation.

Strains that failed to nodulate *L. strigosus* were relatively common, but had no effect on host fitness. In the survey that yielded the strains used in this study, diverse *Bradyrhizobium* strains were isolated from *Lotus* spp. root surfaces, and over half of the root-surface isolates tested came from strains that were unable to nodulate *L. strigosus* in nodulation assays (Sachs *et al.*, 2009). The apparent predominance of non-nodulating strains in natural soils is consistent with previous results from agricultural settings (Segovia *et al.*, 1991; Sullivan *et al.*, 1996; Pongsilp *et al.*, 2002). Clearly, more work is needed to investigate the role of interactions among non-nodulating and symbiotic rhizobia.

To test the hypothesis that symbiotically ineffective rhizobia can be favoured by natural selection, we compared the in-nodule fitness of the symbiotically ineffective strain with that of beneficial strains. We found that within strain variance in rhizobial fitness was large and only the fitness of the symbiotically ineffective strain 2 differed significantly from that of all other genotypes. Strain 2 induced the most nodules per plant. However, they were the smallest on average and produced the least total nodule mass per plant. Such

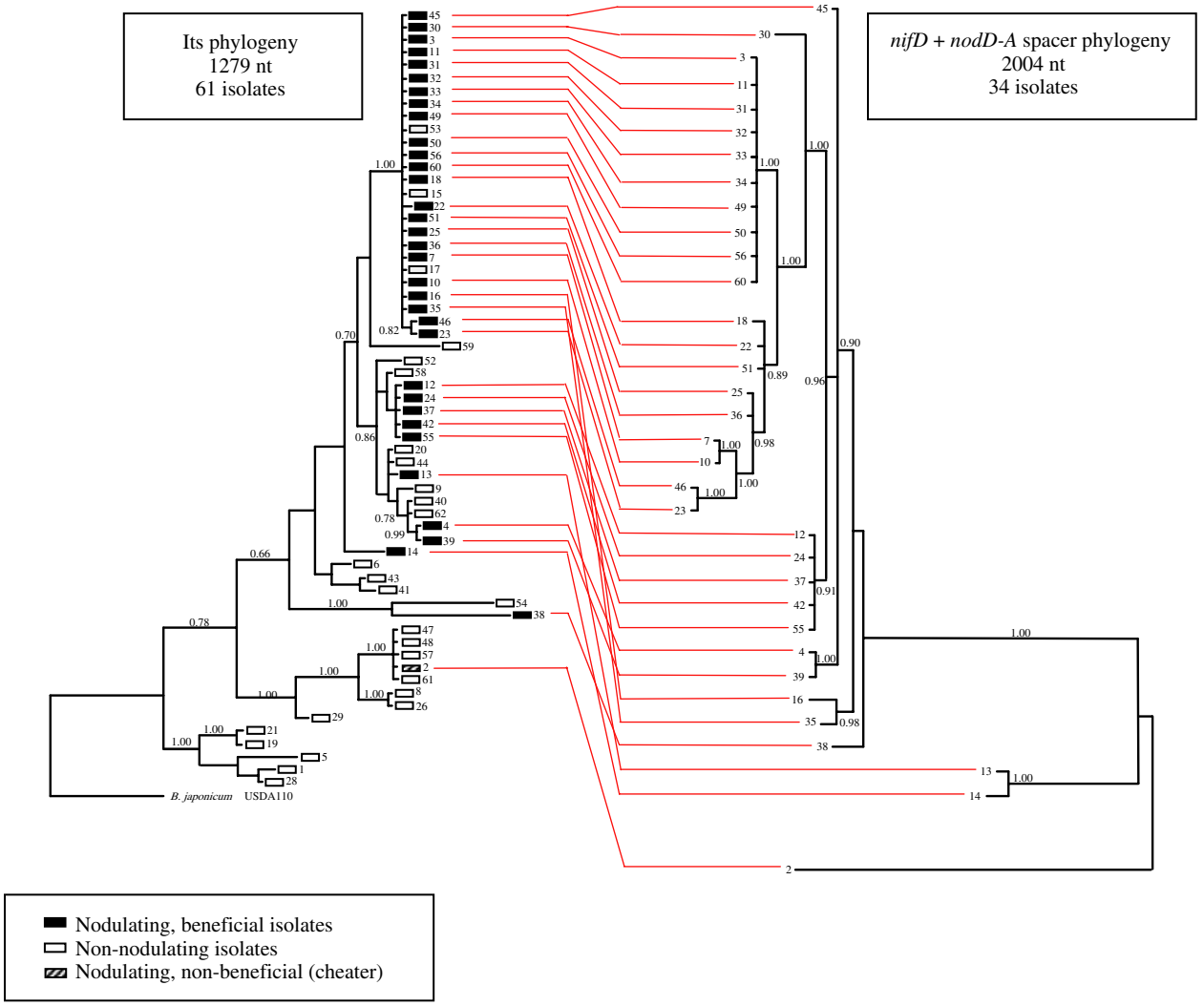

Fig. 3 Bayesian phylogram inferred the *nifD* locus concatenated with the *nodD-A* spacer and mapped as a cophylogeny with the *Its* phylogram. For the *Its* tree, some branches have been rotated, but the topology remains unchanged from Fig. 1. All taxa are labelled with the strain number. Because no suitable outgroup could be found for the *nodD-A* spacer, the *nifD* + *nodD-A* spacer tree was left unrooted. Bayesian clade support values (posterior probabilities) are reported for all well-supported clades (pp ≥ 0.70), and clades with support values ≤ 0.50 are collapsed.

numerous, small nodules are typical of inoculations with nonfixing rhizobia, irrespective of whether the nonfixing phenotype was induced via mutation of a nitrogen fixation gene (Singleton & Stockinger, 1983) or by removing any source of atmospheric nitrogen from around the nodule (Kiers *et al.*, 2003, 2006). Small nodules are often populated by relatively few rhizobia (Kiers *et al.*, 2003; Simms *et al.*, 2006; Heath & Tiffin, 2007). However, there was little or no significant relationship across strains between an individual nodule's mass and its rhizobial population in the sixth-week harvest ($r = -0.249$, $P = 0.09$, $n = 47$) and a weak negative correlation between an individual nodule's size and its rhizobial population in the eighth-week harvest ($r = -0.362$, $P = 0.004$, $n = 62$). Indeed, strain 2 reached population densities per nodule that were fivefold greater than those of the next highest genotype, and more than ninefold greater than the mean for all other strains, consistent with a massive fitness advantage to cheating on hosts. However, because strain 2 did not significantly reduce host fitness, it would not be defined as a parasite.

The proliferation of strain 2 rhizobia in small, symbiotically ineffective nodules conflicts with legume sanctions theory, which predicts that uncooperative rhizobia cannot benefit because they are deprived of host resources by plant sanctioning (Denison, 2000; West *et al.*, 2002). One potential explanation is that the whole-plant fitness advantage exhibited by strain 2 arises only

in a single-inoculation setting. In a more natural setting, we envision two ways that strain 2 might eventually experience negative fitness consequences from cheating. First, legumes can auto-regulate nodule numbers when they have access to sufficient nitrogen, either from fixed or mineral sources (Suzuki *et al.*, 2008). Thus, in a more complex community, *L. strigosus* might obtain sufficient N-fixation capacity from nodules produced by other, more efficient strains. Auto-regulation then might reduce the total number of nodules and hence the number produced by strain 2, thereby limiting the exposure to subversion by strain 2 and reducing the per plant fitness benefit obtained by strain 2. Second, in a more natural setting where plants are subject to many sources of physical and biotic stresses (e.g. drought, herbivores, pathogens, competitors), if a symbiotically ineffective strain was the first to nodulate a seedling, it might be more likely to suffer negative feedback from its negligible contribution to host fitness. Given these possibilities, it will be interesting to examine the fitness benefits accruing to strain 2 in more complex rhizobial communities. This discussion also illustrates the difficulty of identifying cheating. It might be more easily detected in simple situations, such as single-inoculation experiments, but such experiments are also less representative of natural situations.

There are three other potential explanations for why sanctions might not be effective on the symbiotically ineffective rhizobial strain. (i) It might be that *L. strigosus* only rarely encounters cheaters and so has lost or never evolved post-nodulation sanctions mechanisms. If the ability to sanction involves a cost to hosts, it will be maintained only if cheating symbionts are commonly encountered (Foster & Kokko, 2006). We found only one cheater genotype, and we recovered that genotype only once. In contrast, we found multiple cooperative genotypes multiple times (Sachs *et al.*, 2009). (ii) *Lotus strigosus* has sanctioning mechanisms, but the symbiotically ineffective strain could subvert them. This is an interesting possibility, especially considering the novel combination of core-genome and symbiosis island in this strain (Nandasena *et al.*, 2007). (iii) Sanctioning might occur after the stage that we measured, for example during nodule senescence. Hence, even though strain 2 proliferates in nodules it would ultimately be punished before release. This scenario seems unlikely, especially if it is costly for the plant to invest in bacterial growth. However, in the face of our observation that strain 2 obtains a large whole-plant advantage, the first two hypotheses beg for an explanation for the rarity of strain 2.

We examined evidence for fitness trade-offs between rhizobial and plant fitness during infections, which are predicted if rhizobia can maximize fitness by reducing the cooperative resources delivered to the host (Sachs *et al.*, 2004; Foster & Wenseleers, 2006). We found marginally significant negative strain mean correlations of mean nodule number and mean within-nodule population size (both estimates of rhizobial fitness) with inoculation effect on host shoot mass (an estimate of host fitness benefit), suggesting that selection might favour cheating in this system. For most strains, rhizobia produced the largest and most nodules in the largest host plants, consistent with mutual benefit. However for strain 2, nodule size and nodule number varied independently of host growth, which is inconsistent with a mutually beneficial interaction. Strain 2, a wild symbiotically ineffective strain of *Bradyrhizobium*, appears to be a true cheater in the sense that it fails to enhance host growth and nonetheless proliferates within host tissue. More data are necessary to test whether the cheater strain can have superior fitness under more natural conditions.

We found evidence of at least one and likely between two and five evolutionary transitions in which rhizobia that cannot nodulate *L. strigosus* were nested in an ancestrally nodulating clade (Figs 1 and 3). Some of the non-nodulating strains shared 100% sequence similarity with *Its* genotypes of nodulating isolates, consistent with recent loss of nodulation ability on this host. Evidence of evolutionary abandonment of a mutualism has been reported at the species level in phylogenies that encompass large geographic scales (Hibbett *et al.*, 2000; Lutzoni *et al.*, 2001; O'Brien *et al.*, 2005). So far as we are aware, our study provides the first evidence of mutualism abandonment (e.g. Sachs & Simms, 2006) within a population, which is the spatio-temporal level at which natural selection shapes species interactions (Thompson, 2005). Yet an important question is whether the strains that failed to nodulate *L. strigosus* are nonsymbiotic (i.e. lack the ability to infect all host legumes). Our PCR and sequence data can address this issue to a degree. For instance, the failure of the *NifD* primers to amplify only the non-nodulating strains in our study provides evidence that the non-nodulating strains are likely lacking this locus: these exact *NifD* primers and PCR conditions (Parker, 2000) have been used successfully on bradyrhizobia collected from more than 20 diverse host genera over a global range (Australia, China, Costa Rica, Japan, Korea, Mexico, USA) with no failure of amplification in any nodulating isolate (Parker, 2000; Parker *et al.*, 2002; Qian *et al.*, 2003). Furthermore, using the *nodD-A* primers we also found that although all nodulating isolates amplified, including those collected from nodules of two other common host congeners (*L. heermannii*, *L. micranthus*), we were never able to amplify these loci from non-nodulating isolates. The other symbiosis-locus primers, *nifH*, also recovered the same pattern but could not amplify five of the nodulating strains, potentially because of mutations at primer sites, or because this locus is not always required for beneficial symbiosis. These data suggest to us that these evolutionary transitions occurred by wholesale loss of the symbiosis island (and loss of symbiotic ability) rather than by evolution of novel host

specificity. It remains unknown whether these repeated evolutionary transitions are driven by selection or by drift.

In contrast to the evolutionary loss of nodulation ability on *L. strigosus*, we found no evidence consistent with the evolutionary loss or reduction in symbiotic effectiveness within a nodulating clade. The three nodulating isolates that provided little or no benefit to *L. strigosus* were distantly related to beneficial strains and appeared to have independent origins of symbiosis on this host. One of these, strain 14, was difficult to place taxonomically, and in all the post-burnin Bayesian trees was found either within or as sister to one of the beneficial clades described earlier. The other two strains (2, 38) were each placed in separate clades containing strains that did not nodulate *L. strigosus*. Both 2 and 38 appear to have experienced recent horizontal acquisition of symbiosis loci. The horizontal gene-transfer (HGT) hypothesis appears likely because (i) ancestors of each of those genotypes were estimated to be non-nodulating on *L. strigosus* by parsimony and Bayesian methods and (ii) we could not amplify symbiosis-island loci for any of the taxa sister to these genotypes (8, 26, 29, 48, 54, 57, 47, 61), which suggests that they lack the symbiotic island. Moreover, the symbiosis loci are known to be horizontally transmitted (Young & Haukka, 1996; Moulin *et al.*, 2004), whereas the *Its* is not: data from *Its* phylogenies of *Bradyrhizobium* consistently provide congruent results with housekeeping loci (e.g. Wernegreen & Riley, 1999; Moulin *et al.*, 2004). It is possible that HGT events are a common mechanism by which cheaters can emerge within a population of rhizobial mutualists (Sachs & Simms, 2006; Nandasena *et al.*, 2007). More data detailing origins of additional cheating genotypes would be needed to rigorously test this hypothesis.

In conclusion, our study revealed that uncooperative rhizobia – including symbiotically ineffective (nonbeneficial) and non-nodulating strains – coexist in natural populations with beneficial strains. We discovered the first empirical evidence that ineffective rhizobia can exhibit superior fitness compared to beneficial strains. Our phylogenetic reconstruction revealed that non-nodulating strains evolve from mutualist ancestors, potentially via major symbiosis-gene loss events. Finally, we found that ineffective and poorly effective rhizobia can evolve from non-nodulating ancestors, likely via large-scale symbiosis-gene transfer events.

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