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Fitness variation among host species and the paradox of ineffective rhizobia

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Abstract

Legumes can preferentially select beneficial rhizobial symbionts and sanction ineffective strains that fail to fix nitrogen. Yet paradoxically, rhizobial populations vary from highly beneficial to ineffective in natural and agricultural soils. Classic models of symbiosis focus on the single dimension of symbiont cost-benefit to sympatric hosts, but fail to explain the widespread persistence of ineffective rhizobia. Here, we test a novel framework predicting that spatio-temporal and community dynamics can maintain ineffective strains in rhizobial populations. We used clonal and multistrain inoculations and quantitative culturing to investigate the relative fitness of four focal *Bradyrhizobium* strains varying from effective to ineffective on *Acmispon strigosus*. We found that an ineffective *Bradyrhizobium* strain can be sanctioned by its native *A. strigosus* host across the host's range, forming fewer and smaller nodules compared to beneficial strains. But the same ineffective *Bradyrhizobium* strain exhibits a nearly opposite pattern on the broadly sympatric host *Acmispon wrangelianus*, forming large nodules in both clonal and multistrain inoculations. These data suggest that community-level effects could favour the persistence of ineffective rhizobia and contribute to variation in symbiotic nitrogen fixation.

Introduction

Environmentally acquired bacteria provide diverse benefits to plant hosts, including enhanced growth, drought and stress tolerance (Sachs *et al.*, 2010a; Bresson *et al.*, 2013; Antonio Lucas *et al.*, 2014; Coleman-Derr & Tringe, 2014), and improved outcomes in interactions with competitors, herbivores and pathogens (Fravel, 1988; van Loon *et al.*, 1998; Hassan *et al.*, 2010; Friesen *et al.*, 2011; Pieterse *et al.*, 2014). The fitness effects of these bacterial associates often vary widely (Burdon *et al.*, 1999; Heath & Tiffin, 2007; Rodrigues *et al.*, 2008; Sachs & Simms, 2008). For instance, in the legume-rhizobium symbiosis, rhizobial bacteria instigate the formation of nodules on legume roots and fix atmospheric nitrogen for their host in exchange for photosynthates. Rhizobial infections commonly range from highly effective (i.e. enhancing legume growth via nitrogen fixation) to ineffective (i.e. non-nitrogen fixing and not benefiting host growth; Burdon *et al.*, 1999; Sachs *et al.*, 2010a), but the maintenance of this variation is poorly understood.

Legumes can minimize the impact of costly rhizobial infections by discriminating against ineffective rhizobia during nodule formation (partner choice; Devine *et al.*, 1990; Heath & Tiffin, 2009; Sachs *et al.*, 2010b) and by reducing within-nodule growth rates of ineffective rhizobia (sanctions; Kiers *et al.*, 2003; Simms *et al.*, 2006; Sachs *et al.*, 2010b; Oono *et al.*, 2011; Regus *et al.*, 2014, 2015). These host control traits are predicted to lead to the extirpation of ineffective rhizobia (Denison, 2000; Simms & Taylor, 2002; West *et al.*, 2002a, b;

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Sachs *et al.*, 2004; Foster & Wenseleers, 2006). Despite the apparent efficiency of host control by legumes (Regus *et al.*, 2014, 2015), ineffective rhizobia are widespread in natural and agricultural populations (Quigley *et al.*, 1997; Moawad *et al.*, 1998; Burdon *et al.*, 1999; Denton *et al.*, 2000; Chen *et al.*, 2002; Collins *et al.*, 2002; Sachs *et al.*, 2010a). The persistence of ineffective rhizobia remains a paradox with profound impacts on the fitness of wild plants and the success of crops that are enhanced by bacterial symbionts (Triplett & Sadowsky, 1992; Sachs & Simms, 2008; Yates *et al.*, 2011; Friesen, 2012; Heath & Stinchcombe, 2014; Sachs, 2015).

Novel models of symbiosis predict key roles for spatio-temporal variation and host community dynamics in the maintenance of ineffective symbionts (Heath & Stinchcombe, 2014; Steidinger & Bever, 2014, 2016; Bever, 2015). Mutation-selection balance is a useful null model for the maintenance of ineffective rhizobia, wherein ineffective mutants evolve via deleterious mutations but are slowly purged from populations by purifying selection (Van Dyken *et al.*, 2011; Smith *et al.*, 2014). In contrast, spatio-temporal selection models predict scenarios in which ineffective symbionts are maintained in populations above frequencies expected by mutation, including (i) coexistence of effective and ineffective symbionts driven by fitness variation in different host species, (ii) spatially variable selection and (iii) temporally variable selection (Heath & Stinchcombe, 2014). Coexistence of effective and ineffective rhizobia can be maintained if rhizobial fitness varies dependent on the host species, for instance if ineffective rhizobia exhibit a more diverse array of host species than effective genotypes, superior fitness in a subset of host species or superior fitness in the rhizospheres of some hosts (Denison & Kiers, 2004; Sachs & Simms, 2008; Friesen & Mathias, 2010; Sachs *et al.*, 2011). Spatially variable selection models predict that the fitness of a rhizobial genotype varies over space depending on the local host's capacity to reward or sanction that strain (i.e. G × G interactions; Simonsen & Stinchcombe, 2014; Steidinger & Bever, 2014) or depending on spatially variable soil or environmental conditions, [i.e. G × E interactions (Lau *et al.*, 2012)]. Thus, an ineffective rhizobium that has inferior fitness in its sympatric host plant population (e.g. Sachs *et al.*, 2010b; Regus *et al.*, 2014, 2015), might achieve superior fitness as it invades novel host genotypes and or soils. Research has demonstrated that both rhizobial effectiveness and fitness can vary spatially (Burdon *et al.*, 1999; Heath & Tiffin, 2007, 2009; Barrett *et al.*, 2012). But among these studies none has examined spatially variable selection on ineffective rhizobia, which is necessary to examine their maintenance. Finally, selection upon symbionts by host plants could vary temporally within a local population, leading to rhizobial strains exhibiting poor fitness in some years, but superior fitness in other years (Nuismer *et al.*, 2003; Thompson, 2005). Temporal variation could be driven by shifting selection mosaics in co-evolving host–symbiont populations, or if there is negative frequency-dependent selection upon ineffective rhizobia (Bever, 1999; Thompson, 2005; Steidinger & Bever, 2014, 2016).

Here, we investigated the maintenance of ineffective rhizobia in native *Acmispon-Bradyrhizobium* symbioses in California. *Acmispon strigosus* (formerly *Lotus strigosus*; Brouillet, 2008) has been demonstrated to favour effective over ineffective *Bradyrhizobium* genotypes with partner choice and sanctions (Sachs *et al.*, 2010b). The capacity of *A. strigosus* to reward beneficial strains and punish ineffective strains appears robust to wide variation in soil nitrogen and to growth season (Regus *et al.*, 2014, 2015), suggesting that G × E interactions might be less important in the maintenance of variation in this system relative to other sources of fitness variation. We estimated both the fitness effects (upon hosts) and fitness of four focal *Bradyrhizobium* strains that were previously shown to range from effective to ineffective on sympatric *Acmispon* hosts from the Bodega Marine Reserve in Northern California (BMR) (Sachs *et al.*, 2010a). To investigate spatially variable selection among host populations (i.e. G × G), we inoculated hosts gathered from six *A. strigosus* populations across a 700-km transect of California. To investigate fitness variation among host species that could favour ineffective rhizobia, the four *Bradyrhizobium* strains were separately inoculated onto five sympatric *Acmispon* species, including *Acmispon angustissimus*, *Acmispon heermannii*, *Acmispon micranthus*, *A. strigosus* and *Acmispon wrangelianus*. All of these species (except for the non-native *A. angustissimus*) coexist broadly across coastal California and are often syntopic with *A. strigosus* (www.calfora.org). On two close relatives of *A. strigosus*, including *A. heermannii* and *A. wrangelianus* (Allan & Porter, 2000), we also conducted co-infections combining *Bradyrhizobium* genotypes that were previously shown to range from effective to ineffective on sympatric *A. strigosus*. Finally, to examine the potential for temporally variable selection, we analysed previously published inoculation data using the four *Bradyrhizobium* strains on *A. strigosus* seeds from different seed source years at BMR. Subsequently, we conducted a parallel inoculation experiment using *A. strigosus* seeds from six different source years from BMR and compared the fitness and fitness effects of effective and ineffective *Bradyrhizobium* strains.

Materials and methods

Bradyrhizobium strains

Five *Bradyrhizobium* strains, previously referred to as #'s 2, 14, 18, 38 and 49 (Sachs *et al.*, 2010a), were chosen for analyses based upon their natural variation in symbiotic effectiveness on sympatric *A. strigosus* (Sachs *et al.*,

2009, 2010a, b, 2011). In previous experiments, strains #18, #49, #38 and #14 provided a net growth benefit to sympatric *A. strigosus* (increase in shoot biomass relative to uninfected controls) of ~600%, ~%500, ~380% and ~200%, respectively (Sachs *et al.*, 2010a). Strain #2 formed nodules on sympatric *A. strigosus*, but did not enhance the host's growth [i.e. ineffective in this setting (Sachs *et al.*, 2010a)]. Strains #2, #18, #38 and #49 were isolated from *A. strigosus* nodules at BMR, and strain #14 was isolated from *A. micranthus* collected at Sonoma Coast State Park, CA, USA, adjacent to BMR (Sachs *et al.*, 2009). These reserves are coastal, and sampling sites are located in sand dunes with nutrient poor soils (Regus *et al.*, 2014). Phylogenetic reconstruction of the strains showed that effective strains #18 and #49 were nearly genetically identical, and these strains were used interchangeably in previous experiments (Fig. S1; Sachs *et al.*, 2010a, 2011). Thus, we used strain #18 in experiments here, but #49 is also included in some analyses of data from previously published work on seed source year effects. For clarity, the *Bradyrhizobium* strains are hereafter referred to based upon their previously demonstrated per cent growth benefit to sympatric *A. strigosus* hosts (i.e. strains Br600, Br500, Br380, Br200 and Br0; Sachs *et al.*, 2010a) with the understanding that growth benefit could vary substantially among different hosts (a hypothesis that is tested herein).

Collection and preparation of host seeds

Seeds were collected from ripe fruits of each *Acmispon* species. *A. strigosus* seeds were collected from BMR (Lat. 38.319143°N, Long. −123.063657°W) (Sachs *et al.*, 2009), Anza Borrego Desert State Park (33.271264°N, −116.419368°W), Burns Piñon Ridge UC Reserve (34.149309°N, −116.45523°W), a natural site on the UC Riverside campus (33.965938°N, −117.322903°W), the Bernard Field Station in Claremont (34.110525°N, −117.708916°W) and Motte Rimrock UC Reserve (33.804816°N, −117.25802°W). *Acmispon wrangelianus* and *A. heermannii* seeds were also collected from BMR, and *A. angustissimus* and *A. micranthus* seeds were collected from adjacent Sonoma Coast State Park, about 2.5 km away (38.343919°N, −123.057620°W). Seed sets were comprised of equal mixes from multiple parental plants per locale. This approach allows us to study mean host response to a rhizobial genotype within a sampled site (Sachs *et al.*, 2010a, b, 2011; Regus *et al.*, 2014). Seedling preparation followed published protocols (Sachs *et al.*, 2009).

Host inoculation experiments

Four inoculation experiments took place denoted the spatial, species, co-inoculation and temporal experiments. The spatial experiment tested the four focal *Bradyrhizobium* strains (Br600, Br380, Br200 and Br0) and an uninoculated control treatment on the six different *A. strigosus* populations using six replicate plants per treatment combination. Plants were inoculated on 17 December 2013 and were harvested 8 weeks later on 10–12 February 2014. In the species experiment (same greenhouse, inoculation and harvest dates), we tested the four focal *Bradyrhizobium* strains (Br600, Br380, Br200 and Br0) and an uninoculated control treatment on five different *Acmispon* species including, *A. angustissimus*, *A. heermannii*, *A. micranthus*, *A. strigosus* and *A. wrangelianus*, again using six replicate plants. We planned a replication level of ten plants for the spatial and species experiments, but unexpected seedling mortality limited our replication level to the reported six plants per treatment combination. The co-inoculation experiment also took place in parallel. This experiment consisted of 20 plants each of *A. heermannii* and *A. wrangelianus* that were co-inoculated with a 1 : 1 ratio of strain Br0 (ineffective on *A. strigosus*) and Br600 (effective on *A. strigosus*) to test the relative fitness of these strains in the more realistic setting of mixed inocula (where strains can compete for nodulation and the host has the potential to sanction ineffective strains). Multiple published experiments have demonstrated the sanctioning capacity of *A. strigosus* hosts from BMR (on strain Br0), so it is not included here (Sachs *et al.*, 2010a; Regus *et al.*, 2014). No uninoculated controls were used for the co-inoculation experiment as we were not testing hypotheses about host growth response to co-inoculation. Finally, in the temporal experiment, we tested *A. strigosus* whose seeds were collected in different years at BMR, including 2005, 2006, 2007, 2008, 2011 and 2012. These plants were singly inoculated with strain Br0 or Br600 to test whether the relative fitness of the ineffective vs. the most effective strains vary significantly dependent on seed year. Seeds were collected between late May and the middle of June of each year. For most seed sets, we used two spatially separated seed sources per year. For 2012, only one seed set was available. In this case, we doubled the sampling size and randomly split the seed set into two pseudosamples to keep the structure of the experiment similar to the other years. The temporal experiment was divided into five replicated blocks. Each block included 24 plants representing each of the two samples per year. Two seed sets × 6 years × two inoculant strains × five replicate blocks equal a total of 120 plants. Plants were inoculated on 17 April 2015 and were harvested 8 weeks later on 15 to 17 June 2015. No uninoculated controls were used for the temporal experiment.

Bradyrhizobium strains were each grown in liquid media (modified arabinose gluconate, MAG; 29°C, 72 h), and grown cells were washed and resuspended in sterile ddH_2O at the final inoculation density (10^8 cells mL^{-1}; Sachs *et al.*, 2009). Single inoculation treatment plants were inoculated with one of four focal *Bradyrhizobium* strains (5 mL) or with 5 mL of sterilized ddH_2O as the control. In each case, plants were size-

matched by leaf count at the time point of inoculation and randomized for treatment such that inoculated plants were paired with controls of similar size. For the co-inoculation experiment, each plant received an equal mix of strain Br0 and Br600 (5 mL, 2.5×10^8 cells of each strain). For the temporal experiment, plants were inoculated with strain Br0 or Br600 (5 mL; 10^8 cells mL^{-1}).

All plants were fertilized with Jensen's nitrogen-free solution on a weekly basis starting with 1 mL per plant and increasing by 1 mL each week until 5 mL was reached, which was used for the remainder of the experiment. For all experiments, inoculation occurred 6 days after initial fertilization. For all harvests, plants were removed from pots, sand was washed from the roots, and nodules were dissected, counted and photographed. Roots, shoots and nodules were placed into separate paper envelopes and dried in an oven (60°C, > 96 h) before weighing dry biomass. For the co-inoculation experiment, seven plants of each species were randomly selected for nodule culturing to identify and quantify population density of each *Bradyrhizobium* strain within nodules. Culturing of nodules estimates the number of viable rhizobia within each nodule, which can be ultimately released from nodules into the soil (Mergaert *et al.*, 2006; Sachs *et al.*, 2010b; Regus *et al.*, 2014). From each tested plant, we cultured three to four randomly chosen nodules, which were surface sterilized (3% sodium hypochlorite, 2 min, vortexed at least three times), rinsed in sterile ddH_20, three times, macerated with a sterile pestle crushed in sterile ddH_20, and the nodule rhizobia were immediately plated on solid MAG media with replicated serial dilutions of 10^{-3} and 10^{-5} (four plates per nodule). On plates with sufficient growth, at least 100 rhizobial colonies per nodule culture were replica subcultured on an antibiotic treated plate (MAG; streptomycin 100 μg mL^{-1}) and a control plate (MAG media) to estimate the relative proportion of each inoculated strain [Strain Br0 is streptomycin resistant, Br600 is sensitive (Sachs *et al.*, 2010b)]. Previous work suggested no *in vitro* growth rate differences among these strains and also found no evidence of horizontal transfer of antibiotic resistance between strains (Sachs *et al.*, 2010b).

Statistical analysis

We used general linear models (GLM; Fit Model Platform in JMP 10.0 SAS Institute Inc., Cary, NC, USA) to test main effects (rhizobial genotype, host species, host population, host seed source year) and interactions among effects on shoot growth response, shoot biomass, root biomass and plant biomass. To analyse effects of *Bradyrhizobium* inoculation on host growth, we measured shoot, root (minus nodules) and total plant biomass and compared values between inoculated plants and uninoculated controls. To measure symbiotic benefit, we calculated host growth response, which is the mean per cent difference in dry shoot biomass between inoculated plants and un-inoculated control plants. To test whether strains were effective or ineffective on novel hosts, we tested whether shoot growth response differed significantly from zero using a paired *t*-test comparing plant biomass of infected and control plants (JMP 10.0 SAS Institute Inc.; Regus *et al.*, 2014).

To estimate fitness of rhizobial strains within each inoculated host treatment (i.e. *Acmispon* species, *A. strigosus* populations), we compared mean nodule dry biomass and number of nodules, both of which can be positively correlated with rhizobial population sizes in nodules (Sachs *et al.*, 2010a, b; Regus *et al.*, 2014). In previous work, mean nodule biomass was considered a better predictor of rhizobial fitness for two reasons. Firstly, ineffective rhizobia often produce many nodules in isolation, but then are unable to compete for nodulation with beneficial strains under more realistic conditions of multi-strain infections (Sachs *et al.*, 2010a, b; Regus *et al.*, 2014). Secondly, experimental evidence suggests that in single strain inoculations *A. strigosus* can tightly regulate nodule biomass to regulate rhizobial fitness, but that the number of nodules is often a reflection of total root biomass (Regus *et al.*, 2015). To compare fitness among strains on each of the tested hosts, we used pairwise *t*-tests with Tukey's correction for multiple comparisons.

To compare relative nodule biomass caused by each *Bradyrhizobium* strain among diverged hosts (i.e. different species), we generated a relativized scale of nodule biomass values. Nodule biomass values on each host were scaled to a percentile score within each host species (i.e. with 100% representing the largest nodule on each host taxon and 0% represented the smallest). Variance values were scaled accordingly. For the temporal analysis of previously published data (i.e. plant growth, number of nodules, nodule biomass), we analysed *A. strigosus* plants whose seeds were collected in 2005, 2006 and 2011 (the only years that previous data drew from) and which were inoculated with combinations of the five *Bradyrhizobium* strains (Br500 in 2006, Br600 in 2005, 2011, and Br380, Br200 and Br0 in all 3 years). For the analysis of strain fitness in the co-inoculation experiment, we quantified proportional nodule strain populations, defined as the relative population size of the effective strain in plants inoculated by two strains and tested for significance using a chi-square test against a null of 0.50 (the strain inoculation ratio, adjusted via optical density). We report all of our raw data in a supplemental file (Table S1).

Results

Analysis of spatially variable fitness

Measures of relative fitness effects on hosts and relative fitness of the different *Bradyrhizobium* strains were

largely consistent among *A. strigosus* populations. Strain Br600 most often provided the largest growth benefits, followed by strain Br380 and Br200. Strain Br0 never offered significant growth benefit to any host compared to un-inoculated controls and was unable to form nodules on *A. strigosus* from Anza Borrego (Fig. 1). Host biomass, number of nodules and mean nodule biomass all varied significantly dependent upon *Bradyrhizobium* strain and *Acmispon* population source, but there was only a significant strain × host interaction for number of nodules (Table 1). For number of nodules, there were no significant differences among strains upon hosts from BMR, Claremont and Burns Piñon Ridge, and in the other host populations, strain Br600 formed more nodules than the other strains. For mean nodule mass, in all cases strain Br0 formed smaller nodules (or no nodules) compared to all the other strains. In only two populations did any strain form nodules that were significantly larger than Br600: in Anza Borrego and Motte Rimrock, strains Br200 and Br 380, respectively, provided similar growth benefits compared to Br600, but formed significantly larger nodules (Fig. 1). In no case did these data provide evidence that the ineffective strain Br0 experienced superior fitness to any other strains.

Analysis for fitness variation among host species

Host biomass, number of nodules and mean nodule biomass all varied significantly dependent upon *Bradyrhizobium* strain, *Acmispon* species and strain × host interaction (Table 1). Only *A. micranthus* exhibited evidence of nodulation incompatibility, with strains Br600 and Br380 failing to form nodules on most plants (Table S1). For aboveground host growth, two host species exhibited no significant growth effects from inoculation (*A. angustissimus*, *A. wrangelianus*), and one host species only received growth benefit from strain

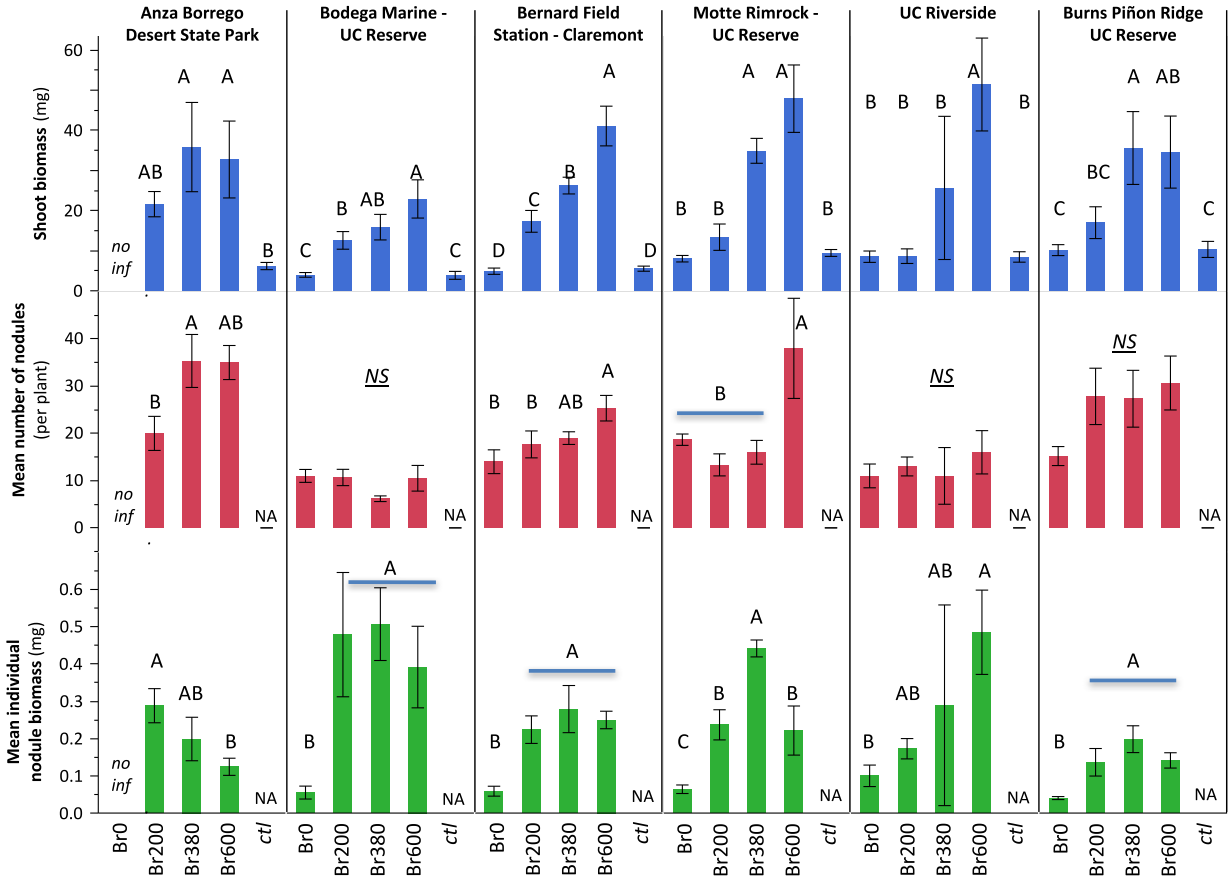

Fig. 1 Effects of *Bradyrhizobium* inoculation on *Acmispon strigosus* from populations across their range in California. Shoot biomass, number of nodules and nodule mass are compared among four *Bradyrhizobium* strains upon six *A. strigosus* populations. For host growth, we compared shoot biomass of infected *Acmispon* spp. to uninoculated controls (*ctl*). Within each host population, letters represent levels that differ significantly using a pairwise *t*-test with Tukey's correction for multiple comparisons ($P < 0.05$). Error bars show one standard error above and below the mean. Hosts from Anza Borrego did not become nodulated by strain Br0 (*no inf.*) and uninoculated controls never became nodulated (NA).

Table 1 Effects of *Bradyrhizobium* genotype and host source on fitness measures.

Host	Fixed effect	DF	Host biomass†	Number of nodules†	Mean nodule biomass†
Acmispon spp.	Strain	3	7.89***	26.69***	18.11***
	Host	4	45.38***	6.24***	45.42***
	Strain × Host	12	7.72***	18.07**	13.11***
Acmispon strigosus populations	Strain	4	40.06***	39.78***	22.96***
	Host	5	3.82*	10.30***	5.79***
	Strain × Host	20	1.28	3.42***	1.69
Acmispon strigosus years	Strain	3	16.86***	10.16***	31.71***
	Host	3	11.34***	40.76***	13.43***
	Strain × Host	9	1.22	9.98***	1.87

*$P < 0.01$, **$P < 0.001$, and ***$P < 0.0001$.

†F ratio from least square fit linear model for fixed effects of *Bradyrhizobium* genotype, host genotype and the interaction among these factors.

Br200 (*A. micranthus*; Fig. 2). For *A. heermannii* and *A. strigosus*, Br600 caused the largest growth benefit, followed in order by strains Br380, Br200 and Br0. In no case did strain (Br0) provide significant growth benefits to any of these hosts.

The ineffective strain Br0 formed larger nodules than any of the other strains on *A. wrangelianus*, even though it provided no benefits to the host (Figs 2 and 3). In contrast, Br0 formed significantly smaller nodules on *A. micranthus* and on *A. heermannii* and with no significant differences among strains on *A. angustissimus*. On *A. micranthus*, strain Br200 formed the largest nodules (Figs 2 and 3). For mean number of nodules, there was either little or no difference among strains for *A. angustissimus* and *A. strigosus*, respectively. On *A. micranthus*, the ineffective strain Br0 formed the most nodules, but not significantly more than strain Br200, and the other two strains formed few or no nodules. On *A. wrangelianus* and *A. heermannii*, only strain Br600 differed significantly by forming more nodules than all the other strains (Fig. 2).

Co-inoculation results

Acmispon wrangelianus hosts inoculated with equal proportions of strains Br0 and Br600 were dominated by strain Br0. Strain Br0 exhibited a mean of ~96% population proportion in the subcultured nodules (Fig. 4). However, only 9 of 26 nodules were successfully cultured. All of the nodules were small, white and irregularly shaped and most showed signs of initial senescence, wherein the bleach sterilization step can be lethal to rhizobia. Total *Bradyrhizobium* population sizes of *A. wrangelianus* nodules were $\sim 3 \times 10^5$. Thus, co-inoculations of *A. wrangelianus* suggested that Br0 has superior fitness on this hosts when compared to Br600 in this setting. In contrast, *A. heermannii* hosts co-inoculated with equal proportions of strains Br0 and Br600 were in every case co-infected with both strains (all tested nodules). Strain Br600 had a mean of ~75% of the population within the co-infected nodules, significantly more than the 50% null expectation, which would assume random nodule formation (Fig. 4). Eight *A. heermannii* nodules were not successfully cultured and appeared to be in the initial stages of nodule senescence. These data suggest that *A. heermannii* can efficiently reward the beneficial strain Br600 and can sanction the ineffective strain Br0, consistent with multiple experiments on the close relative *A. strigosus* (Sachs *et al.*, 2010b; Regus *et al.*, 2014). Total *Bradyrhizobium* population sizes of *A. heermanii* nodules were consistent with other studies, with a mean nodule population size of $\sim 3 \times 10^6$ (Sachs *et al.*, 2010a, b).

Analysis for temporal variation in fitness

Comparing published data sets for the effects of *Bradyrhizobium* infection on host fitness, there were no differences among seed source year in the relative benefit of the strains, with strains Br600 and Br500 providing the most growth benefit, followed in order by Br380, Br200 and Br0 (Fig. S2). In all cases, strain Br0 provided no growth benefits to hosts. For mean number of nodules, there was little or no difference among strains in seeds from 2011 to 2006, respectively. However, for seeds from 2005 strain Br0 formed more than 2× the number of nodules than any other strain. For mean nodule mass, in all three seed source years strain Br0 formed smaller nodules compared to all the other strains (Fig. S2).

For our temporal seed set experiment with strains Br600 and Br0, there were significant differences among *A. strigosus* seed sets, with strain Br0 forming significantly more nodules in one of the 2005 seed sets and Br600 forming significantly more nodules in one of the 2006 seed sets (2005.1, $F_{1,6} = 14.79$, $P = 0.009$; 2006.2, $F_{1,8} = 5.697$, $P = 0.0441$; Fig. S3). In all other seed sets, there were no significant differences in number of nodules between strains Br600 and Br0. For mean nodule mass, in all seed sets strain Br0 formed smaller nodules compared to Br600 ($F_{1,112} = 217.72$, $P < 0.0001$; Fig. S3). These analyses do not allow us to support or reject the hypothesis that temporally variable selection can maintain ineffective rhizobia.

Discussion

We investigated the maintenance of ineffective rhizobia in native *Acmispon-Bradyrhizobium* symbioses in California by employing four focal *Bradyrhizobium* strains, including one isolate that was ineffective on all tested hosts. We found evidence that the ineffective *Bradyrhizobium* strain (Br0) interacts with *Acmispon* taxa in a

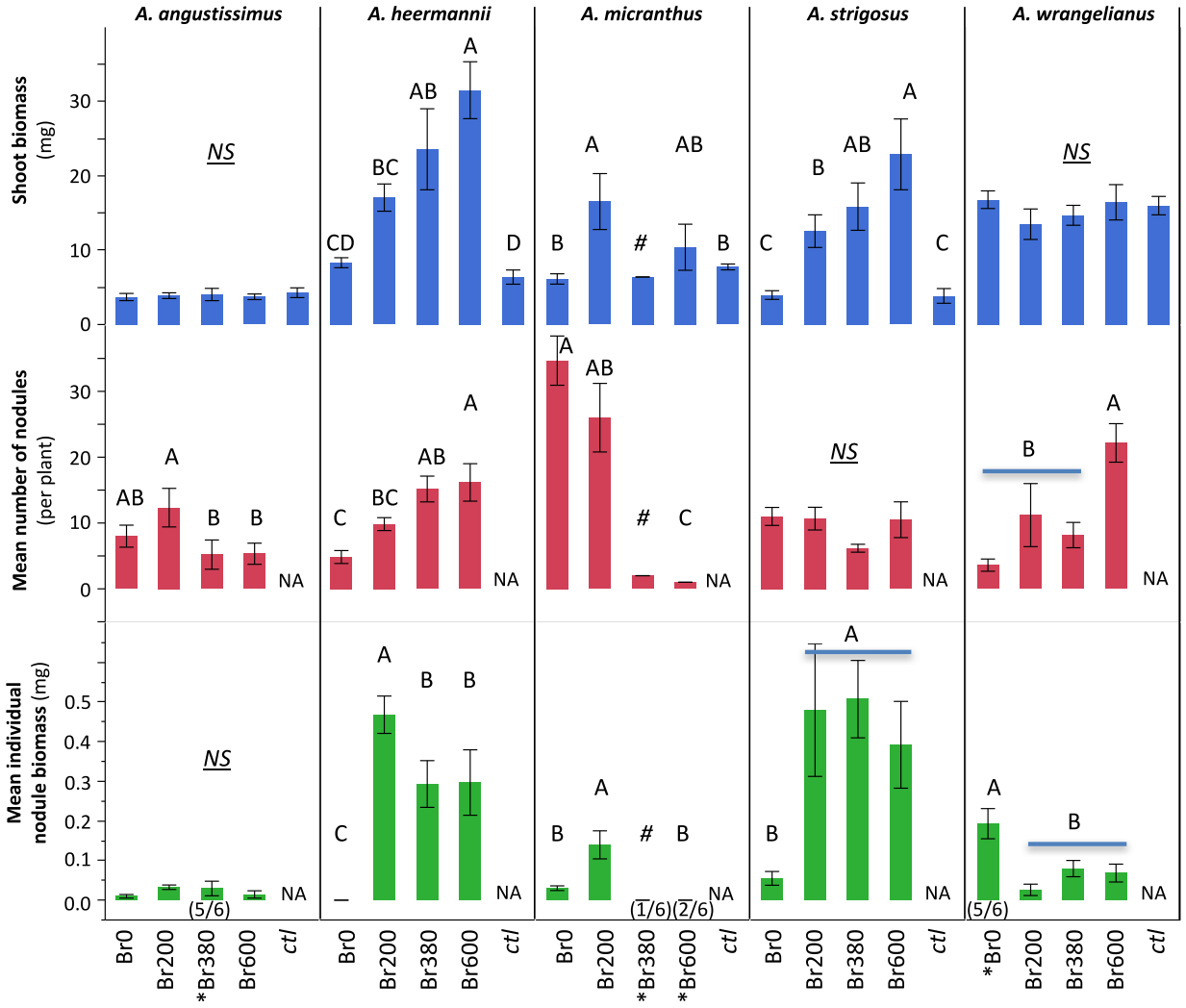

Fig. 2 Effects of *Bradyrhizobium* inoculation on different *Acmispon* species. Shoot biomass, number of nodules and nodule mass are compared among four focal *Bradyrhizobium* strains upon five *Acmispon* species. For host growth, we compared shoot biomass of infected *Acmispon* spp. to uninoculated controls (*ctl*). Within each host species, letters represent levels that differ significantly using a pairwise *t*-test with Tukey's correction for multiple comparisons ($P < 0.05$), and letters are not indicated if only one datapoint exists for a treatment (#). Error bars show one standard error above and below the mean. Asterisks indicate strain–host combinations where some inoculated hosts remained uninfected (zero nodules). When a subset of inoculated hosts formed nodules, the fractions of hosts that formed nodules are indicated in parentheses. Uninoculated controls never became nodulated (NA).

host species-dependent manner. Our clonal inoculation experiments suggest that there is significant fitness variation for Br0, wherein it forms smaller and fewer nodules than other strains on *A. strigosus* hosts across the native host's range (Fig. 1), but forms significantly larger nodules than all other strains on *A. wrangelianus* (Figs 2 and 3). Multistrain infection experiments further support this pattern in that strain Br0 was sanctioned by its native host *A. strigosus* (Sachs *et al.*, 2010b) and by the congener *A. heermannii* (Fig. 4), thus inhabiting nodules at only low frequencies when in competition with other strains. In contrast, Br0 dominated nodule occupancy in mixed strain inoculations on *A. wrangelianus* (Fig. 4). Demonstrating that ineffective strain Br0 can gain access to larger nodules and can be competitively dominant to strain Br600 on *A. wrangelianus* could be ecologically relevant because (i) strain Br600 is the most beneficial strain that we have yet studied (Sachs *et al.*, 2010a), (ii) *A. wrangelianus* is a sister taxon to *A. strigosus* (Allan & Porter, 2000), (iii) these host species exhibit an overlapping range throughout much of California (www.calflora.org) and often grown right next to each other (Fig. S4), and (iv) *Bradyrhizobium* persist in the

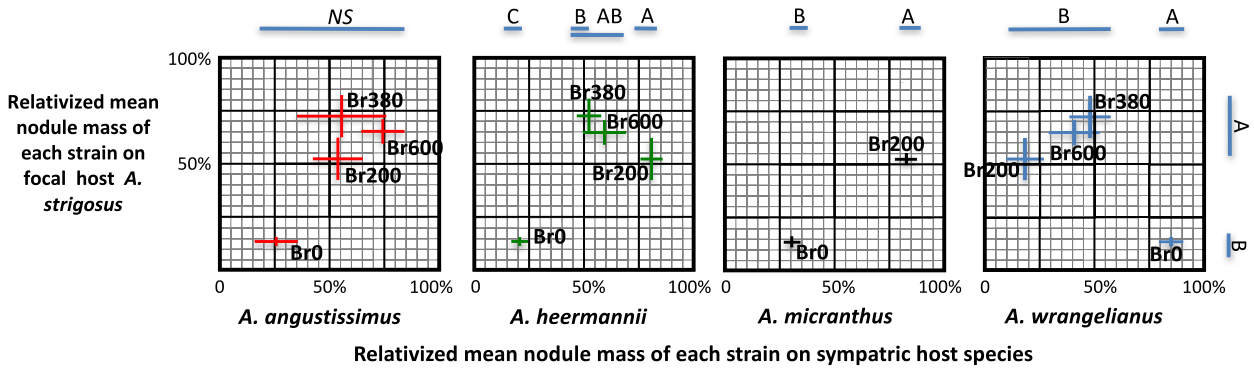

Fig. 3 Relative nodule biomass measures of different *Bradyrhizobium* strains in sympatric *Acmispon* species. Mean nodule biomass per plant for each *Bradyrhizobium* strain is shown on a relativized scale within each host species and compared between the focal host, *Acmispon strigosus*, and four other species, *A. angustissimus*, *Acmispon heermannii*, *Acmispon micranthus* and *Acmispon wrangelianus*. Error bars show one standard error above and below the mean for the relativized values. For each species but *A. wrangelianus*, the ineffective strain Br0 has the smallest mean nodule biomass. On *A. micranthus*, only strains Br0 and Br200 consistently formed nodules. Within each host species, letters represent levels that differ significantly using a pairwise *t*-test with Tukey's correction for multiple comparisons ($P < 0.05$).

rhizosphere of *A. wrangelianus*, even though this host primarily associates with *Mesorhizobium* (Sachs *et al.*, 2009). The data from nodule biomass suggest to us that the ineffective strain Br0 experiences inferior fitness on the hosts species of origin (*A. strigosus*) but might experience superior fitness on the closely related sister taxon (*A. wrangelianus*) at least when in competition with the *Bradyrhizobium* strains in question. However, the data set for the numbers of nodules formed on *A. strigosus* and *A. wrangelianus* showed the opposite result (more nodules for the beneficial strain Br600), so this question remains unresolved. Similar evidence of host-dependent fitness variation was also uncovered among related *Bradyrhizobium* that can nodulate *A. strigosus* and *Lupinus bicolor*, but in that case ineffective strains always showed evidence of inferior fitness to effective strains (Ehinger *et al.*, 2014). Overall, our data suggest that Br0 could be maintained because of fitness variation among the tested host species, but it is unclear whether evidence from this one strain has implications on ineffective rhizobia more generally.

Our experiments do not resolve whether Br0 could be competitive or even persist on *A. wrangelianus* under natural conditions. One possible scenario for the maintenance of Br0 is that it occasionally nodulates *A. wrangelianus* and forms large nodules when it does, potentially because *A. wrangelianus* has no mechanism to reward or punish atypical symbionts (Sachs *et al.*, 2009). Formation of large nodules is thought to be correlated with repopulation of the soil with large numbers of rhizobia, but this correlation remains poorly understood. To test hypotheses for the maintenance of Br0 on *A. wrangelianus*, it would be necessary to compete Br0 against *Mesorhizobium* strains that typically nodulate *A. wrangelianus*. Moreover, if strain Br0 does experience marked host-dependent fitness variation, these conditions have not apparently led this strain to reach high frequencies across a wide geographic range like some *Bradyrhizobium* strains have on *A. strigosus* (Hollowell *et al.*, 2016). To better resolve the spatial array of fitness variation among host plant species, it would be key to compare independent fitness measures of these *Bradyrhizobium* strains (and other effective and ineffective strains) on multiple host taxa at each of the tested field sites, and with more replication than presented here.

We uncovered significant differences in number of nodules dependent on host and symbiont combinations. In particular, the ineffective strain Br0 varied significantly in relative number of nodules (compared to beneficial strains) among *A. strigosus* hosts from different seed source years. Both experimental data herein and previously published data sets suggest that strain Br0 forms significantly more nodules than the beneficial strain(s) on some *A. strigosus* hosts sourced from 2005 (Figs S2 and S3). However, nodule biomass of strain Br0 was much smaller than all other strains and total nodule biomass produced by strain Br0 was also significantly less than in other strains (Figs S2 and S3). Thus, with mixed results we are not able to confidently support or reject temporally variable selection upon the ineffective strain Br0. We also found a trend that ineffective strain Br0 forms more nodules on *A. micranthus* than the other strains. But only the strains Br200 and Br0 were able to consistently nodulate *A. micranthus*, and in this case, strain Br0 did not form significantly more nodules than Br200 (Fig. 2). Moreover, plants with strain Br0 had significantly less total nodule biomass than plants inoculated with strain Br200 ($F_{1,19} = 7.22$, $P = 0.0019$), suggesting that the relative fitness of strain Br0 might be inferior if competition experiments were undertaken. As discussed above, data

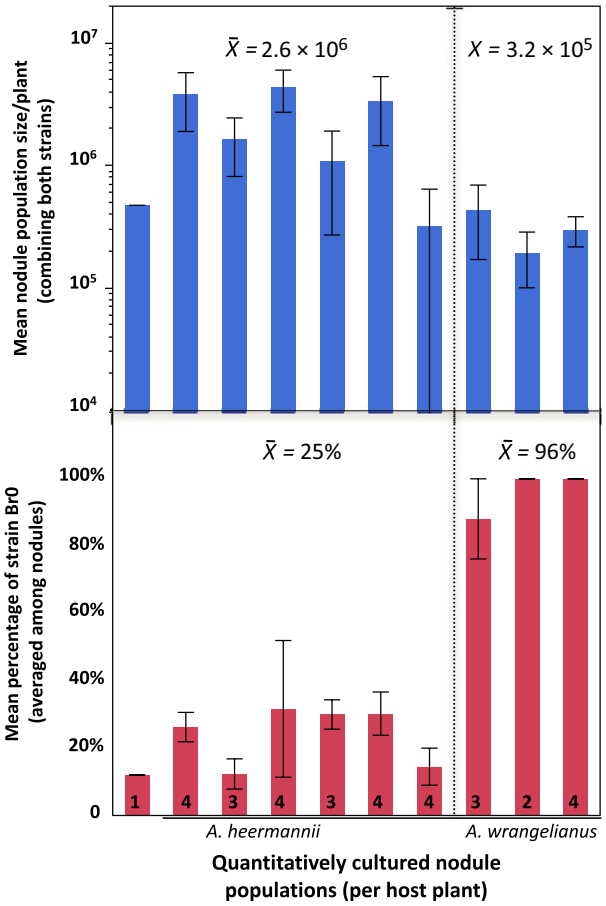

Fig. 4 *Bradyrhizobium* fitness in *Acmispon heermannii* and *Acmispon wrangelianus* sanctions experiment. *Bradyrhizobium* fitness is shown as total population size of rhizobia per nodule for each host species ~ (top panel), and the relative proportion of strain Br0 in each nodule as estimated via colony subculturing (bottom). Error bars show one standard error above and below the mean in both. Number of nodules cultured is shown by the bold numbers. Each bar represents data from the nodules of one plant replicate.

on number of nodules are considered cautiously by many researchers because ineffective strains often form many nodules on plants in isolation, but are unable to compete with other rhizobia for nodulation (Friesen & Mathias, 2010; Sachs *et al.*, 2010b; Regus *et al.*, 2014). We consider these data as preliminary and needing further work to substantiate, perhaps with larger experiments that focus on addressing temporal fitness variation, rather than the broader approach used here to test multiple hypotheses.

We found mixed evidence for spatially variable selection among *A. strigosus* populations. However, for the ineffective strain Br0, we found that it formed fewer and smaller nodules than the other *Bradyrhizobium* strains in almost all *A. strigosus* populations. Thus, although there were significant differences among *A. strigosus* genotypes in their investment into different symbionts, as has been found by other researchers (Simonsen & Stinchcombe, 2014; Haney *et al.*, 2015), no *A. strigosus* host population appeared to favour Br0 over the effective genotypes. Among host populations, only plants from Anza Borrego differed from the other sites in that these hosts were resistant to strain Br0 and did not form significantly larger nodules with strain Br600 compared to the other strains. The strongest evidence for spatially variable selection came from strains that provided relatively low benefits to hosts, such as Br200 and Br380, but that experienced evidence of superior fitness in Anza Borrego and Motte Rimrock, respectively (Fig. 1). Given the subtle differences in the fitness and fitness effects, it would be challenging to resolve spatially variable selection among strains such as Br200 and Br 380. Previous research has found unequivocal evidence of G × G interactions among legume host populations (Heath & Tiffin, 2007; Barrett *et al.*, 2012). However, it could be that there was more standing genetic variation in those hosts compared to our *Acmispon* populations. *Acmispon strigosus* self-pollinates in the greenhouse, has cleistogamous flowers and has seeds that fall via gravity without carriage by other animals, all suggesting that this species might be genetically depauperate (Hamrick & Godt, 1996).

Hypotheses to explain the maintenance of variation in symbiotic effectiveness need not be mutually exclusive (Heath & Stinchcombe, 2014). We tested three models in our study, but our hypothesis testing approach was not exhaustive. For instance, our approach does not allow us to rule out mutation-selection balance (Van Dyken *et al.*, 2011; Smith *et al.*, 2014), which would occur if ineffective mutants are regularly introduced into populations but get purified by selection. Our fitness data do not support mutation-selection balance given the evidence that ineffective strains formed larger nodules to effective rhizobia in some settings. Yet population genetic data from this and related lineages of rhizobia have suggested a form of mutation-selection balance based upon horizontal transfer events. Unlike the typical mutation-selection model, in which point mutations are thought to be the main driver of phenotypic change, ineffective rhizobia can be generated by horizontal gene transfer events of symbiosis island loci into novel chromosomal backgrounds (Nandasena *et al.*, 2007; Sachs *et al.*, 2010a, 2011). The evolution of ineffective rhizobia in these cases is likely driven by negative epistasis between symbiosis island and chromosomal loci (Sachs *et al.*, 2011). Moreover, we used an approach here of testing several hypotheses simultaneously and thus with some compromise in power of testing any hypothesis with greater replication.

An emerging framework for plant-associated symbioses predicts key roles for spatio-temporal variation and host community dynamics in the maintenance of

ineffective symbionts (Thompson, 2005; Thompson & Fernandez, 2006; Heath & Stinchcombe, 2014; Bever, 2015). Our results suggest that fitness variation among host species might promote ineffective strains in a host species-dependent manner. These data might also be useful to make predictions about legumes in agricultural settings, where ineffective rhizobia can occur at high frequencies. Research over the past 50 years has characterized 1000s of rhizobial strains that are effective under greenhouse conditions (i.e. they fix nitrogen, substantially enhance plant growth), but to date, most of these strains have not proved successful as field inocula (Triplett & Sadowsky, 1992; Yates *et al.*, 2011; Sachs *et al.*, 2013). When candidate rhizobia are inoculated into crops, they are out-competed by indigenous soil rhizobia that produce mediocre yields (Tang *et al.*, 2012). This long-standing challenge is termed the 'rhizobial competition problem' (Triplett & Sadowsky, 1992; Yates *et al.*, 2011). Our data suggest that plant community context could be a driver of ineffective infections in legume crops, especially as these crops are often dominantly nodulated by the symbionts of locally indigenous legumes irrespective of the strains that are inoculated onto the crops (Tang *et al.*, 2012). Thus, it is key to design inoculation programmes that consider the multiple drivers of rhizobial fitness including local soil conditions, host diversity (both crop and wild) and the population of competing symbionts.

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Author contributions

V.J.P., P.J.N.S, A.C.H., J.U.R., K.A.G., C.E.W., K.W.Q., J.Y.L. and J.L.S. performed experiments, J.U.R. and J.L.S. conducted fieldwork, V.J.P. and J.L.S. analysed data and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Phylogenetic relationships among referenced strains.

Figure S2 Effects of seed source from past experiments; *Bradyrhizobium* inoculation on sympatric *Acmispon strigosus* from different seed years.

Figure S3 Temporal experiment; *Bradyrhizobium* inoculation on sympatric *Acmispon strigosus* from different seed years.

Figure S4 Photos of *Acmispon strigosus* and *Acmispon wrangelianus* growing syntopically.

Table S1 Raw data on biomass of nodules, roots, and shoots.

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