

Mutualists Stabilize the Coexistence of Congeneric Legumes

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ABSTRACT: Coexistence requires that stabilizing niche differences, which cause species to limit themselves more than others, outweigh relative fitness differences, which cause competitive exclusion. Interactions with shared mutualists, which can differentially affect host fitness and change in magnitude with host frequency, can satisfy these conditions for coexistence, yet empirical tests of mutualist effects on relative fitness and stabilizing niche differences are largely lacking within the framework of coexistence theory. Here, we show that N-fixing rhizobial mutualists mediate coexistence in four naturally co-occurring congeneric legume (*Trifolium*) species. Using experimental greenhouse communities, we quantified relative fitness and stabilizing niche differences for each species in the presence of rhizobia originating from conspecific or congeneric hosts. Rhizobia stabilized coexistence by increasing the self-limitation of *Trifolium* species grown with rhizobia isolated from conspecifics, thus allowing congeners to increase when rare. Greenhouse-measured invasion growth rates predicted natural, unmanipulated coexistence dynamics of *Trifolium* species over 2 years at our field sites. Our results demonstrate that interactions with shared mutualists can stabilize the coexistence of closely related species.

Keywords: coexistence, mutualism, negative frequency dependence, plant-soil feedback, rhizobia.

Introduction

The mechanisms underlying species coexistence are at the core of biodiversity and an enduring question in ecology. According to modern coexistence theory, stable coexistence requires that stabilizing niche differences, which cause species to limit themselves more than others, outweigh relative fitness differences, which cause competitive exclusion (Chesson 2000b; Levine and Hille Ris Lambers 2009). Stabilizing

niche differences can arise from mechanisms such as resource partitioning (Hutchinson 1959; MacArthur 1970; Tilman 1982), differing responses to spatial and temporal environmental variation (Chesson 1994, 2000a), and specialized interactions with natural enemies (Janzen 1970; Connell 1971). Stabilizing mechanisms result in negative frequency dependence of species' growth rates (Adler et al. 2007; Levine et al. 2008) such that species have an advantage when rare, allowing each to recover from low densities and thus enabling coexistence (MacArthur 1972; Chesson 2000a). Recent studies have found negative frequency dependent performance in plant communities (Harpole and Suding 2007; Godoy and Levine 2014; Chung and Rudgers 2016), demonstrating the existence of stabilizing niche differences. However, relatively few studies have simultaneously explored both critical components of coexistence—relative fitness differences and stabilizing niche differences—or explored how they are influenced by specific ecological interactions (but see Godoy et al. 2017; Mayfield and Stouffer 2017).

It is becoming increasingly clear that soil microbial communities can play important roles in plant species interactions and coexistence (Van der Heijden et al. 2008; Bever et al. 2012; Van der Putten et al. 2013). Plants interact with a diverse set of soil microbes, including enemies such as bacterial and fungal pathogens and mutualists such as mycorrhizal fungi and, in the case of legumes, nitrogen-fixing rhizobial bacteria. These interactions can generate plant-soil feedbacks (PSFs) when changes in the soil microbial community caused by a plant species feed back to influence the relative performance of the species and its heterospecific neighbors (Bever 1994; Van der Heijden et al. 2008; Van der Putten et al. 2013). PSFs can influence relative plant species performance directly by providing different amounts of benefit or harm to different species or indirectly by altering the relative strength of interspecific and intraspecific competition (Casper and Castelli 2007; Pendergast et al. 2013).

Stabilizing effects promoting coexistence can also arise through mutualists if mutualists fostered by one species provide more benefit to that species' competitors than to itself (Bever et al. 1997; Bever 1999; Van der Heijden et al.

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2008) or alter plant species interactions in ways that increase self-limitation. While this possibility has been most extensively explored in PSFs, it applies to any mutualisms in which mutualists provide different fitness benefits to multiple partners and in which relative benefits may also be altered by the relative frequency of species in the community (as modeled in Bever 1999; Umbanhowar and McCann 2005; Steidinger and Bever 2014). Despite the plethora of PSF studies, these feedbacks are rarely directly tested in the context of coexistence models. We know of only one study that has experimentally tested the effects of PSFs on negative frequency dependence in plant communities. Chung and Rudgers (2016), working with two arid-land grasses, found that pathogenic rhizosphere soil microbes increased the strength of negative frequency dependence in the competitively dominant species, thus stabilizing coexistence. The role of mutualists in stabilizing the coexistence of multispecies communities remains untested.

Here, we examine the role of nitrogen-fixing rhizobial mutualists (*Rhizobium leguminosarum* biovar *trifolii*) in the coexistence of four native annual congeneric legume (*Trifolium*) species that exhibit long-term local co-occurrence in grassland communities in northern California. As close relatives, these species have similar habitat preferences, resource requirements, and enemies (Parker and Gilbert 2007), making their coexistence difficult to explain through traditional niche-partitioning mechanisms (Darwin 1859; Wiens et al. 2010; Weber and Strauss 2016). They also share mutualistic partners, a prerequisite for stabilizing negative net feedbacks through mutualism in coexistence models (Bever 1999; Umbanhowar and McCann 2005; Steidinger and Bever 2014). Our ongoing work shows that strains isolated from one *Trifolium* species will nodulate with many other *Trifolium* species but provide different benefits across hosts, thus making this an ideal system for exploring the role of mutualists in coexistence.

We focus on four questions: First, do *Trifolium* species perform better in the presence of rhizobia isolated from congeneric vs. conspecific hosts, indicating negative rhizobially mediated PSF? Second, how does rhizobial origin (conspecific vs. congeneric) influence the strength and direction of frequency dependence in *Trifolium* growth and nodulation? Third, how does rhizobial origin influence the ability of *Trifolium* species to increase when rare, the key criterion for stable coexistence? And fourth, do these dynamics predict patterns of historical coexistence and invasion when rare in natural *Trifolium* communities? We answered these questions using a greenhouse experiment in which we grew *Trifolium* species at five frequencies (11%–100%) at biologically realistic field densities in the presence of rhizobia isolated from field-grown nodules of either conspecific or congeneric *Trifolium*. Using the results of this experiment, we quantified the strength and direction of rhizobially mediated PSFs and

their role in *Trifolium* frequency dependence (questions 1 and 2). We then used neighborhood competition models to quantify the effects of rhizobial origin on relative fitness differences, stabilizing niche differences, invasion growth rates, and coexistence outcomes of *Trifolium* species pairs (question 3). Finally, we related these results to observed coexistence patterns observed directly from natural communities (question 4).

Material and Methods

Study System

We studied the coexistence of four species of *Trifolium* in coastal grasslands at the University of California Bodega Marine Reserve (BMR), Bodega Bay, California (38°15'81"N, 123°4'9"W). *Trifolium barbigerum*, *T. bifidum*, *T. macraei*, and *T. microdon* are annual legumes that associate with the nitrogen-fixing bacterium *Rhizobium leguminosarum* (biovar *trifolii*). These common species, along with *T. fucatum*, *T. microcephalum*, *T. wildenovii*, and *T. gracilentum*, co-occur at fine spatial scales in coastal grasslands. The deeper-soiled portions of the coastal prairie are dominated by exotic annual grasses, but as the soil thins toward the top of the coastal bluffs, native species become more prominent. As a group, *Trifolium* species comprise a major component of the native plant community in these areas, occupying 2%–50% cover across a 20–40-m-wide band of habitat that runs the length of the coastal bluffs (A. Siefert, K. W. Zillig, M. L. Friesen, S. Y. Strauss, G. Gilbert, and I. Parker, unpublished data from field surveys). Our data, combined with those of Parker and Gilbert (2007) collected in the same areas, show that these species have co-occurred in the same habitats at fine spatial scales (within 4-m² plots) over the past 15 years; we thus interpret their interactions as true coexistence. From our field surveys, 68% of monitored plots with *Trifolium* had two or more *Trifolium* species in them, and 32% had one species.

Greenhouse Experiment

To understand the role of rhizobia in *Trifolium* species coexistence, we conducted a greenhouse experiment in which we grew the four focal species in mixed communities while manipulating conspecific frequency and rhizobial origin. Because rhizobia are very small and cannot be independently manipulated from other small microbial soil components, controlled inoculations are the only way with which to manipulate rhizobial strain associations with *Trifolium* hosts. The experimental design was a replacement series in which plants were grown in communities of 36 individuals at a constant density (approximately 1,000 m⁻²), similar to *Trifolium* density in natural communities observed in field

surveys at BMR. Replacement series designs have been criticized for inaccurately predicting coexistence if species' relative performance is density dependent and experiments are conducted at densities far from the equilibrium densities of the competing species (Damgaard 2008). However, Levine et al. (2008) found that replacement series designs conducted at densities close to those of natural communities, such as our experiment, are effective at quantifying the strength of frequency dependence and stabilizing niche differences.

Focal species were grown at five frequencies: 11%, 33%, 56%, 78%, and 100%. Remaining individuals in the community were evenly distributed among the three congeneric species. Growing species across this frequency gradient allowed us to estimate the slope of the relationship between each species' performance and its frequency in the community, a measure of the strength of stabilizing niche differences—a steeper negative slope indicates stronger stabilization. This frequency gradient was replicated for each focal species in two rhizobial origin treatments: conspecific and congeneric. Rhizobial strains were isolated from nodules of plants of each species collected from the field; thus, for example, strains isolated from *T. barbigerum* were considered conspecific strains for *T. barbigerum* plants but congeneric strains for *T. microdon* plants and so on. Rhizobial community composition is likely to reflect that of the *Trifolium* community and vary over time. We assume that the conspecific rhizobial treatment represents what a *Trifolium* species largely experiences when it is abundant, while the congeneric rhizobial treatment represents what a *Trifolium* species likely experiences when it is rare.

Our previous work has isolated rhizobial strains and found that most strains will nodulate with all annual *Trifolium* species and that, for two of the species investigated, strains did not preferentially nodulate across species (Siefert et al. 2018b). While nodulation rates did not differ by host species, benefits conferred to *Trifolium* host species by rhizobial strains do vary across these host species (C. A. Friel, S. Y. Strauss, and M. L. Friesen, unpublished data). Thus, congener-origin strains can nodulate with the focal *Trifolium* species but may not confer similar benefits.

Conspecific rhizobia treatments consisted of a mix of three rhizobial strains isolated from nodules of the focal species (for details of rhizobium strain isolation, see app. A; apps. A, B are available online). As 68% of our field quadrats have two or more *Trifolium* species, we used a mixed congeneric inoculum as a realistic simulation of field conditions. Thus, congeneric rhizobial communities were a mixture of three strains, one strain originating from each of the three congeneric competitor species. PSFs are usually calculated between species and soil communities cultured by individual species; here, we assume that our congeneric rhizobial treatment (encompassing rhizobia from three different species) captures average rhizobially mediated interactions between

the focal species and its three competitors. This design kept the number of strains in each treatment constant (three conspecific or three congeneric), which we felt was important.

For each inoculum treatment type (conspecific or congeneric rhizobia), we created two unique three-strain mixture replicates, A and B. Replicate strain mixtures within rhizobial origin treatments contained completely different rhizobial strains isolated from different field nodules. Replicates were included as random effects in our analyses to account for mean strain differences. In sum, we conducted a 4 species \times 5 frequency \times 2 rhizobial origin factorial experiment with two rhizobial strain mixtures per origin, resulting in 80 experimental communities consisting of 2,880 individuals. We grew an additional six individuals per focal species and rhizobial strain mixture alone (one individual per pot) to assess the performance of plants in the absence of competition. Overall, this design allowed us to compare the performance of each species with its own versus its competitors' rhizobia, with the recognition that congeneric strains were sourced from three species.

Planting and Inoculation

Experimental communities were established in doubled-up 18.5 \times 18.5 \times 8.5-cm plastic containers filled to a depth of 6 cm with potting medium. High walls prevented splashing during watering. Potting medium, consisting of a 50:50 mix of potting mix (Sunshine Mix no. 1, Sungro Horticulture Canada, Bellevue, WA) and sand. The nutrient profile and chemistry of the potting medium was similar to that of field soil at BMR (table B1; tables B1–B7 are available online), ensuring realistic growing conditions. Potting medium was sterilized by autoclaving twice at 15 lb of pressure and a temperature of 120°C for 30 min.

Seeds from 36 maternal families per focal species, originating from field collections at BMR and subsequently propagated in the greenhouse for at least one generation, were randomized and planted into containers in a 6 \times 6 grid with individuals spaced 2.5 cm apart. Positions of individuals were randomized within communities except for four individuals of the focal species designated for nodule sampling (see below), which were nonrandomly placed in positions not on edges and not sharing neighbors. Individuals that did not survive 2 weeks after planting were replaced with extra greenhouse-germinated seedlings.

Each seedling received 2 mL of rhizobial inoculum (10^7 cells mL⁻¹) applied by pipette in two doses of 1 mL at 1 and 3 weeks after planting. After 10 weeks, we harvested, dried, and weighed shoots of all individuals. In addition, we collected roots of four focal individuals per community for nodule measurements and Sanger sequenced *nodC* of replicate nodules to establish rhizobial strain identities (for methods, see app. A). To check our inoculation methodol-

ogy, we Sanger sequenced the *nodC* locus of each inoculum strain (for methods, see app. A); while not all strains can be uniquely identified with *nodC*, we were able to find differentiated strains and track them across experimental treatments. On the basis of 119 assignable nodules, nodule strain occupancy was correct with respect to our treatments 84% of the time. Moreover, uninoculated plants in the greenhouse become chlorotic and eventually died. Thus, we have demonstrated that contamination across treatments is low; furthermore, any cross contamination would serve to weaken the effects of rhizobial origin, and therefore our estimates are conservative.

Roots were cleaned, scanned, oven dried, and weighed. We counted and measured the one-sided projected area of nodules using ImageJ. Aboveground biomass was highly correlated with total biomass of focal plants (overall $r = 0.93$), confirming its usefulness as a measure of overall plant performance. We estimated seed production in this experiment from shoot biomass using species-specific regressions we generated from plants grown under similar greenhouse conditions for which all seed was collected ($r = 0.70$ – 0.78 ; table B2). Greenhouse experiment data are deposited in the Dryad Digital Repository: <https://dx.doi.org/10.5061/dryad.1f2c124> (Siefert et al. 2018a).

Data Analysis

We examined the interactive effects of rhizobial origin and frequency at two levels: community and neighborhood. In the community-level analysis, we examined the per capita growth and nodulation of each focal species as a function of its frequency in the community and rhizobial origin, thus allowing us to address our first question, Do *Trifolium* species perform better in the presence of conspecific versus congeneric rhizobia, indicating negative rhizobially mediated PSF? This analysis also allowed us to estimate the overall strength frequency dependence for each species, which reflects the net effects of interactions between the focal species and multiple congeneric competitors, thus allowing us to address our second question, How does rhizobial origin influence the strength and direction of frequency dependence in *Trifolium* growth and nodulation? In the neighborhood-level analysis, we examined the performance of focal species individuals as a function of the density of conspecific and congeneric competitors in their immediate neighborhoods. Using neighborhood competition models, we estimated the strengths of inter- and intraspecific competition and equalizing and stabilizing effects for each species pair in the presence of the focal species' conspecific and congeneric rhizobia. This analysis provided additional information about the pairwise species interactions underlying overall patterns of frequency dependence seen at the community level and allowed us to address our third ques-

tion, How does rhizobial origin influence the ability of *Trifolium* species to increase when rare?

Community-Level Analysis

We quantified the relative performance of focal species with conspecific versus congeneric rhizobia, an index of PSF mediated by rhizobial strain identity (question 1), using the following formula:

$$\text{PSF index} = \ln\left(\frac{\text{biomass}_{\text{conspecific}}}{\text{biomass}_{\text{congeneric}}}\right),$$

where $\text{biomass}_{\text{conspecific}}$ and $\text{biomass}_{\text{congeneric}}$ are the per capita shoot biomass of the focal species in the presence of conspecific and congeneric rhizobia, respectively. Positive values indicate positive feedback (greater biomass with conspecific vs. congeneric rhizobia), and negative values indicate net negative feedback (greater biomass with congeneric vs. conspecific rhizobia). Since there were two replicates of each rhizobial origin treatment and previous authors have recommended against calculating PSF effects using treatment means (Brinkman et al. 2010), we calculated rhizobial feedback effects by randomly pairing each conspecific rhizobial replicate with a congeneric rhizobial replicate within focal species and frequency levels. We examined the effects of focal species, frequency, and their interactions on the strength and direction of rhizobial feedbacks using a model selection approach (see above). The global model included focal species, frequency, and their interactions as fixed effects and rhizobial strain mix as a random effect. Examination of the data showed that rhizobial feedbacks differed strongly between the lowest frequency (11%) and all higher frequencies, and this threshold effect was not well described by a linear or quadratic relationship. We subsequently treated frequency as a binary variable, representing either low (11%) or high (>11%) frequency.

We examined the effects of frequency and rhizobial origin on focal species' mean community-level growth and nodulation using a model selection approach. For each response variable (per capita aboveground biomass, nodule number, and mean nodule size), we fit a linear mixed model with focal species, frequency, rhizobial origin, and all two- and three-way interactions as fixed effects and rhizobial strain mix as a random effect. Preliminary analysis showed that allowing the slope of the frequency effect to vary among rhizobial strain mixes within rhizobial origin treatment did not significantly improve model fit (likelihood ratio test, $P = .18$), so we did not include this random effect in subsequent models. We then fit a set of models that included all possible subsets of fixed effects present in the full model, ranked the models using the corrected Akaike information criterion, and calculated model probabilities (Akaike weights) using the MuMIn package in R (Bartoń 2013). Support for models including

a frequency-by-rhizobial origin effect would indicate that rhizobial origin influences the strength and/or direction of frequency dependence in *Trifolium* performance (question 2).

Neighborhood-Level Analysis

We assessed the effects of rhizobial origin on pairwise species coexistence outcomes (question 3) by comparing the invasion growth rate and its component terms—relative fitness inequality and stabilization—for each focal species invading each congener in the presence of the focal species' conspecific or congeneric rhizobia (similar to approaches used in Godoy et al. [2017], but in our case also in the context of rhizobial treatments). A greater invasion growth rate in the presence of congeneric rhizobia indicates that rhizobial feedbacks enhance the ability of a species to increase when rare, promoting coexistence (specified in eq. [2] below).

To estimate invasion growth rates, we first modeled the performance of focal species individuals as a function of conspecific and congeneric neighbor densities using an annual plant competition model (Watkinson 1980):

$$F_{pirm} = \frac{\lambda_{irm}}{1 + \sum_{j=1}^s \alpha_{ijr} N_j}, \quad (1)$$

where F_{pirm} is the seed production of individual p of species i in rhizobial treatment r (conspecific or congeneric rhizobia) and rhizobial strain mix m , λ_{irm} is the mean per capita seed production (number of seeds) of species i in rhizobial treatment r and rhizobial strain mix m in the absence of neighbors, α_{ijr} is the per capita effect of species j on species i (including the effect of species i on itself, α_{ii}) in rhizobial treatment r , and N_j is the number of individuals of species j in the neighborhood of the target plant. We defined the neighborhood as the eight grid positions adjacent to the target plant and included "edge" as a neighbor category to account for effects of growing on edges of pots. Because we harvested the experiment before all plants had set seed, we estimated seed production from shoot biomass using species-specific regressions from plants grown under similar greenhouse conditions ($r = 0.70$ – 0.78 ; table B2). We calculated λ s as the mean per capita seed production of individuals grown alone in each rhizobial inoculum. The remaining model parameters were fit by maximum likelihood estimation, assuming a normal error distribution, using the mle2 function in R (Bolker 2016). We fit separate models for each species with conspecific and congeneric rhizobia.

Next, we assessed coexistence for each species pair by estimating the per capita rate of increase of one species when it is rare and the other species is at its equilibrium density—that is, the invasion growth rate—using the following equation (Adler et al. 2007):

$$\text{IGR}_{ijr} = \frac{\lambda_{ir}}{\lambda_{jr}} \left[\frac{\lambda_{jr}}{1 + (\alpha_{ijr}/\alpha_{jrr})(\lambda_{jr} - 1)} \right], \quad (2)$$

where λ_{ir} and λ_{jr} are the per capita seed production of the invading and resident species, respectively, in the absence of competition in rhizobial treatment r (the invaders' conspecific or congeneric rhizobia), averaged across replicate strain mixtures, and IGR_{ijr} is the growth rate of focal species i with resident species j in rhizobial treatment r . For stable coexistence of two species, both species must have an invasion growth rate that exceeds 1. In this equation, $\lambda_{ir}/\lambda_{jr}$ represents the relative fitness difference in the absence of stabilizing effects. The bracketed term represents the strength of stabilization (Adler et al. 2007), which is determined by the degree to which intraspecific competition (α_{jrr}) exceeds interspecific competition (α_{ijr}). Invasion growth rates cannot be calculated using this model if interspecific or intraspecific facilitation occurs (i.e., negative α). We did not observe intraspecific facilitation of any of our species, and in rare cases of weak interspecific facilitation we set α_{ij} equal to 0. In such cases, the invasion growth rate equals the focal species' intrinsic rate of increase (λ_i).

We assume that coexistence metrics calculated with congeneric rhizobia treatment parameters are most relevant to invasion growth rates when rare (for each rare invader invading a common resident). We compared coexistence metrics calculated with congeneric rhizobia treatment parameters to those calculated with conspecific rhizobia treatment parameters to gain an understanding of the role that congeneric rhizobia play in coexistence. We acknowledge that complex temporal dynamics in rhizobial communities, influenced by variation in annual *Trifolium* communities over time, could also influence coexistence in ways that might not be captured by these metrics. We also note that this model assumes perfect seed survival and germination and thus may overestimate invasion growth rates. Nevertheless, the model allowed us to assess coexistence as determined by postgermination neighborhood interactions under different rhizobial origins and could be further supported or refuted by observed field dynamics, as described below.

We tested whether invasion growth rates and their component terms (relative fitness inequality and stabilization) differed between conspecific and congeneric rhizobia treatments across each pairwise invasion scenario involving the four *Trifolium* species (hereafter, "invasions"; $n = 12$) using permutation tests. For invasion growth rates, relative fitness differences, and stabilization, we calculated the mean difference between conspecific and congeneric rhizobia treatments averaged across all pairwise invasions and then compared this to a null distribution generated by permuting rhizobial treatments within invasions 1,000 times to calculate a two-sided P value.

Coexistence in Natural Communities

We were interested in testing whether invasion growth rates, relative fitness inequalities, and stabilizing effects measured in our greenhouse experiment could predict invasion dynamics of *Trifolium* species in the field (question 4). To estimate the invasion dynamics of *Trifolium* species in natural communities, we recorded *Trifolium* species presence in five hundred sixty-three 0.09-m² plots at BMR in April 2015 and 2016. For each species, we modeled presence in 2016 as a function of presence of conspecifics and congeners in 2015 using logistic regression with a quasi-binomial error distribution. While these models do not directly test coexistence and are not directly analogous to invasion growth rate estimates from our greenhouse experiment, a positive year-to-year effect of species *j* on the presence of species *i* (hereafter, “field invasibility index”) suggests that species *i* is able invade and persist in plots occupied by species *j*, indicating the potential for stable coexistence. To test whether these interactions were explained by invasion dynamics quantified in our greenhouse experiment, we tested the correlation between field invasibility index and greenhouse-measured invasion growth rates, relative fitness differences, and stabilizing effects across invasion scenarios ($n = 12$) using permutation tests (as above).

Results

Effects of Rhizobial Origin on *Trifolium* Frequency Dependence: Community-Level Analyses

Analysis of mean *Trifolium* species performance growing in communities that varied in frequency of the focal species showed that the strength and direction of frequency dependence varied among species and between rhizobial origins

(the best model included frequency \times rhizobial origin and frequency \times focal species interactions; $R^2 = 0.65$; Akaike weight, 0.38; fig. 1; table B3), indicating that rhizobially mediated PSFs altered *Trifolium* frequency dependence. However, we note that the second best model included rhizobial origin and the frequency \times focal species interaction but not the frequency \times rhizobia interaction (Akaike weight, 0.309; table B3). Overall, species experienced marginally negative frequency dependence in the presence of rhizobia isolated from congeneric hosts (slope, -0.0014 ; 95% confidence interval [CI], -0.003 to 0.000) but had frequency-independent performance in the presence of rhizobia from conspecific hosts (slope, 0.0006 ; 95% CI, -0.001 to 0.002). The strength and direction of frequency dependence varied among species. *Trifolium macraei*, the largest species, exhibited negative frequency dependence (slope, -0.004 ; 95% CI, -0.007 to -0.002). In contrast, *T. bifidum*, the smallest species, exhibited positive frequency dependence (slope, 0.0035 ; 95% CI, 0.001 to 0.006). *Trifolium barbigerum* and *T. microdon* had frequency-independent performance. Three of the four species (all except *T. bifidum*) performed best at low frequency and in the presence of congeneric rhizobia (fig. 1), the conditions they would face as invaders into a community of congeners. Congeneric rhizobia treatments represent a mix of strains from three different species, so it is unclear whether negative and positive feedbacks arose due to interactions with just one, all, or a subset of congeners.

Nodulation with Conspecific and Congeneric Rhizobia

All sampled focal individuals in both conspecific and congeneric rhizobia treatments produced nodules, confirming that rhizobial strains were shared among species; nodule

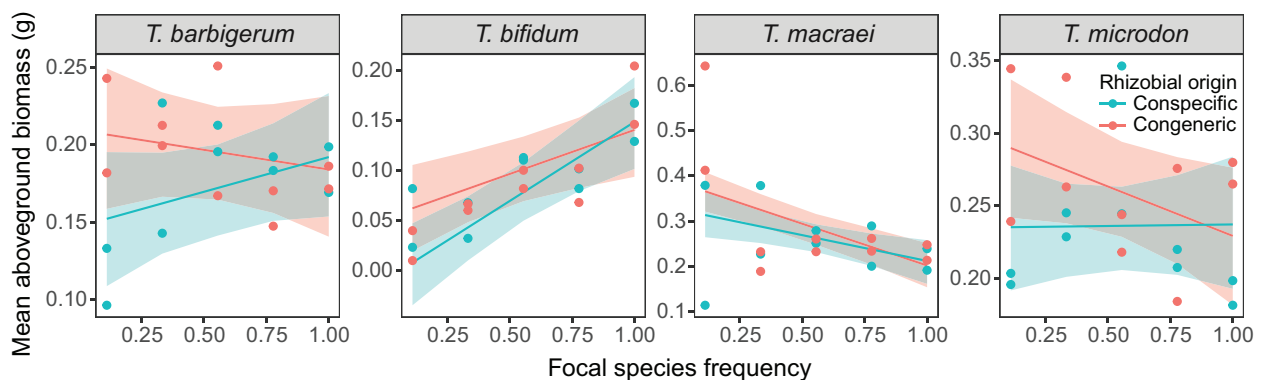


Figure 1: Frequency-dependent performance of *Trifolium* species as a function of rhizobial identity. When a species is rare, it is most likely to experience congeneric rhizobia; thus, when a focal species (indicated at the top of each graph) is at low frequency and has been inoculated with congeneric rhizobia, the data represent the invasion scenario. Conversely, a focal species at high density with conspecific rhizobia reflects the situation when it is the resident. Data are from 36-individual *Trifolium* communities ($n = 80$) in a greenhouse experiment where rhizobia either originated from conspecific or congeneric field nodules. Gray bands indicate 95% confidence intervals on linear mixed model predictions (best model determined by corrected Akaike information criterion model selection).

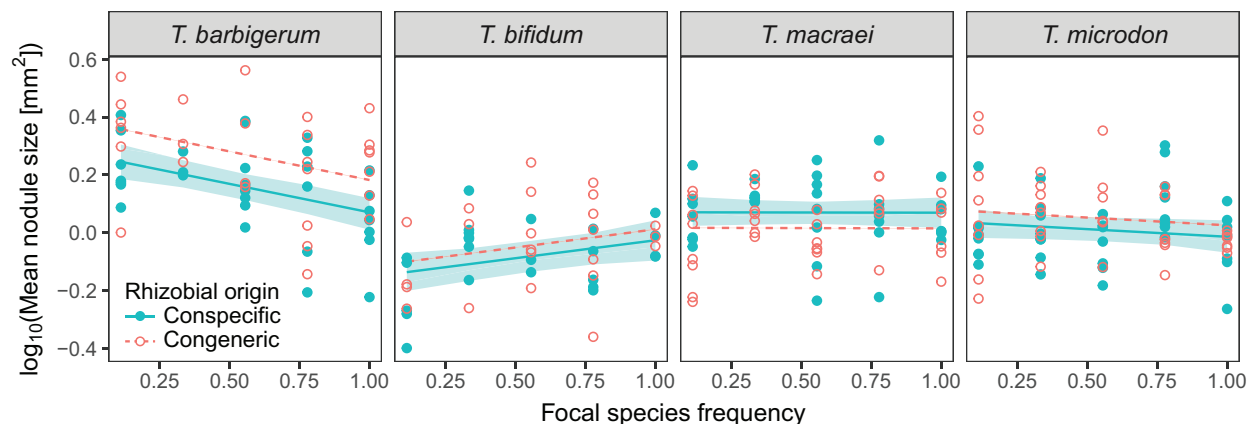


Figure 2: Frequency-dependent nodule size of *Trifolium* species as a function of rhizobial identity. Shown is the mean nodule size of *Trifolium* individuals ($n = 250$) in the presence of rhizobia isolated from conspecific or congeneric hosts in 36-individual *Trifolium* communities in a greenhouse experiment. Gray bands indicate 95% confidence intervals on linear mixed model predictions (best model determined by corrected Akaike information criterion model selection).

genotyping further confirmed that our rhizobial treatments were effective. For all species, biomass was positively related to total nodule number ($R^2 = 0.66$) and, to a lesser extent, mean nodule size ($R^2 = 0.29$). Effects of rhizobial origin and frequency on mean nodule size varied among focal species (fig. 2; table B4). *Trifolium barbigerum* produced larger nodules at low frequencies and in the presence of congeneric rhizobia (fig. 2); in contrast, *T. bifidum* produced smaller nodules when it was rare (fig. 2), consistent with its positive frequency-dependent growth. We did not find support for effects of frequency or rhizobial origin on total nodule number per individual (fig. B1; table B5). We note that since we did not conduct a fully factorial inoculation

design, we were not able to account for overall differences in inoculum effectiveness. However, we do not observe overall differences in inoculum effectiveness for plants grown alone or under completely conspecific competition, suggesting that the inoculum mixes do not vary substantially in their overall ability to promote plant growth.

Rhizobially Mediated PSF

We found significant effects of rhizobial origin on the mean performance of focal species within communities, indicating rhizobially mediated PSFs (fig. 3). The strength and direction of rhizobial PSF depended on focal species and fre-

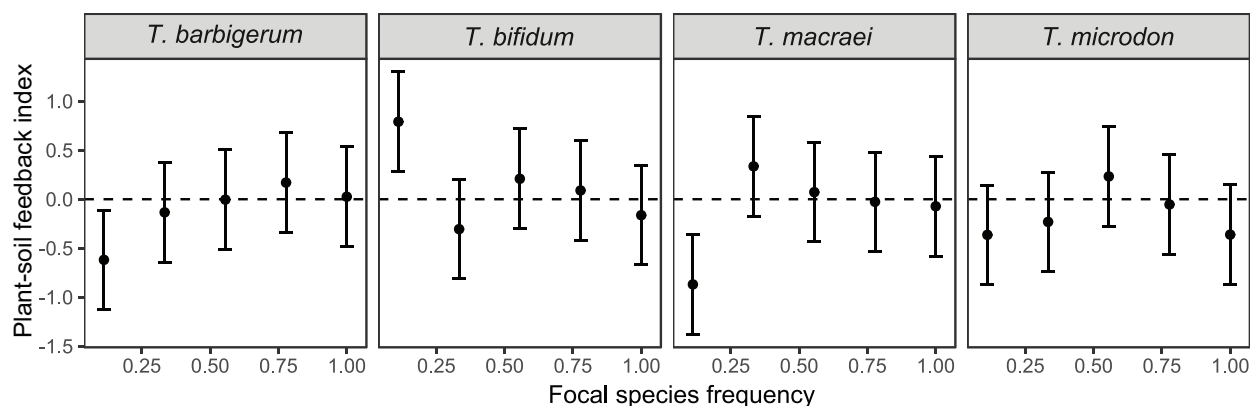


Figure 3: Frequency-dependent plant-soil feedback of *Trifolium* species. Shown is plant-soil feedback for *Trifolium* species grown at different frequencies (11%–100%) in 36-individual *Trifolium* communities ($n = 80$) in a greenhouse experiment. Plant-soil feedback was calculated as the natural logarithm of the ratio of mean aboveground biomass in the presence of rhizobia isolated from conspecific versus congeneric hosts. Positive values indicate positive rhizobially mediated plant-soil feedback (higher biomass with conspecific vs. congeneric rhizobia); negative values indicate negative feedback (higher biomass with congeneric vs. conspecific rhizobia). Error bars show 95% confidence intervals on linear mixed model predictions.

quency (the model with the highest support included the frequency \times focal species interaction; $R^2 = 0.49$; Akaike weight, 0.98; table B6). When grown at low frequency (11%), two of four species (*T. barbigerum* and *T. macraei*) had significant negative rhizobial PSF, that is, higher biomass with congeneric than conspecific rhizobia. In contrast, one species (*T. bifidum*) had positive rhizobial PSF when grown at low frequency. At frequencies above 11%, rhizobial PSFs were neutral for all species. Rhizobial PSFs were also neutral for all species in the absence of competition. This indicates that PSFs mediated by rhizobial mutualists were important predominantly when species were rare.

Effects of Rhizobial Origin on *Trifolium* Frequency Dependence: Neighborhood Analyses

We used neighborhood competition models (table B7) to assess the effect of rhizobial origin on the ability of focal species to increase when rare in the presence of each congener, the key criterion for stable coexistence. The effect of rhizobial origin depended on the *Trifolium* species pair (fig. 4). Overall, the mean invasion growth rate across the 12 pairwise invasion scenarios was greater in the presence of congeneric than conspecific rhizobia (permutation test, $P = .03$), indicating that rhizobially mediated PSF enhanced the ability of *Trifolium* species to increase when rare. Invasion growth rates greater than 1 were predicted for seven of 12 invasions

with congeneric rhizobia (fig. 4A), compared with only three invasions with conspecific rhizobia (fig. 4B). Mutual invasibility, indicating stable coexistence, was predicted for three of six species pairs (*T. microdon* and each of its congeners) when the invading species was with congeneric rhizobia, whereas no species pairs were predicted to coexist when the invading species was with conspecific rhizobia (fig. 4).

By examining the components of invasion growth rates (relative fitness inequality and stabilization), we found that rhizobial interactions influenced coexistence primarily by enhancing stabilizing effects, that is, the degree to which intraspecific competitive effects of resident species exceed interspecific competitive effects. Stabilizing effects were greater in the presence of the invading species' congeneric rhizobia than conspecific rhizobia (permutation test, $P = .014$), indicating that resident species had stronger self-limitation in the presence of their own rhizobia. Stabilization was predicted for eight of 12 invasions with congeneric rhizobia (fig. 4A), compared with three invasions with conspecific rhizobia (fig. 4B). In contrast, invading species had lower frequency-independent relative fitness (relative fitness difference term < 1) with congeneric than conspecific rhizobia (permutation test, $P = .009$). In other words, rhizobially mediated PSF increased the relative fitness inequality between invaders and resident species, but this effect was outweighed by increased stabilization through self-limitation by the resident species. The overall result was a positive net ef-

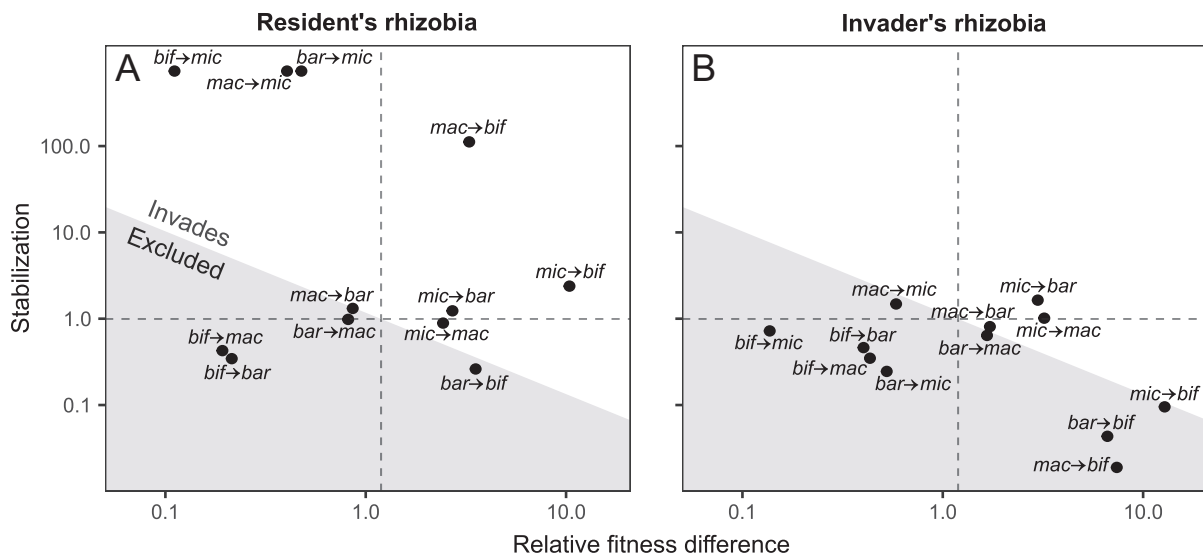


Figure 4: Rhizobial identity affects predicted *Trifolium* species coexistence. Depicted are the results of the coexistence analysis for *Trifolium* species, showing model-predicted relative fitness difference and stabilizing effects for each focal species invading each congener in the presence of rhizobia isolated from the congeners (A) or from the focal species (B). Relative fitness difference values > 1 indicate that the focal species has higher average fitness than the resident in the absence of stabilizing effects. Stabilization values > 1 indicate stabilizing effects (intraspecific competitive effect of resident exceeds interspecific competitive effect; i.e., self-limitation). Gray areas indicate invasion growth rates < 1 (focal species is excluded by the resident), and white areas indicate invasion growth rates > 1 (focal species increases when rare with the resident at equilibrium density). Relative fitness differences and stabilization values are shown on a log₁₀ scale for clarity. bar = *T. barbigerum*; bif = *T. bifidum*; mac = *T. macraei*; mic = *T. microdon*.

fect of rhizobial PSF on invasion growth rates and coexistence. We note that since we did not conduct a fully factorial inoculation design, we were not able to account for overall differences in inoculum effectiveness. However, we do not observe overall differences in inoculum effectiveness for plants grown alone or under completely conspecific competition, suggesting that the inoculum mixes do not vary substantially in their overall ability to promote plant growth.

Greenhouse-Measured Invasion Growth Rates Predict Local Coexistence in Field Trifolium Assemblages

Invasion dynamics quantified in the greenhouse experiment explained species coexistence dynamics in natural *Trifolium* assemblages at our study site (Bodega Bay, CA). Field invasibility, estimated as the effect of a species' presence in the previous year on the presence of the focal species in the next year within 0.09-m² plots, was positively related to greenhouse-measured invasion growth rates of focal species in the presence of congeneric rhizobia ($r = 0.50$; permutation test, $P = .04$; fig. 5A). When looking at the components of coexistence—relative fitness differences and stabilizing niche differences—we found that field invasibility was marginally positively related to both the relative fitness advantage of the focal species ($r = 0.40$; $P = .11$) and the strength of stabilizing effects ($r = 0.33$; $P = .15$) in the presence of congeneric rhizobia. Field invasibility was not significantly correlated with green-

house-estimated invasion growth rates, relative fitness differences, or stabilizing effects in the presence of conspecific rhizobia (fig. 5B).

Discussion

Mutualist-mediated coexistence dynamics can occur when shared mutualists provide different fitness benefits across species and when benefits also change as a function of species' frequency in the community (e.g., Runquist and Stanton 2013; Schmid et al. 2016; Guitian et al. 2017; Mayfield and Stouffer 2017). Interactions with mutualists have received far less attention than either resource competition or host/pathogen-based feedbacks in coexistence (but see Bever 2002; Castelli and Casper 2003), but there is growing evidence that mutualists could drive coexistence dynamics (Runquist and Stanton 2013; Schmid et al. 2016; Guitian et al. 2017). Here, we highlight the role of soil *Rhizobium* bacteria in stabilizing the coexistence of four congeneric legume (*Trifolium*) species through PSFs. Using greenhouse experiments and field observations, our study shows that mutualists may mediate the coexistence of species that share them and that these effects hinge on host frequency within a community.

Coexistence is determined by the balance between relative fitness differences, which lead to competitive exclusion, and stabilizing niche differences, which promote the ability

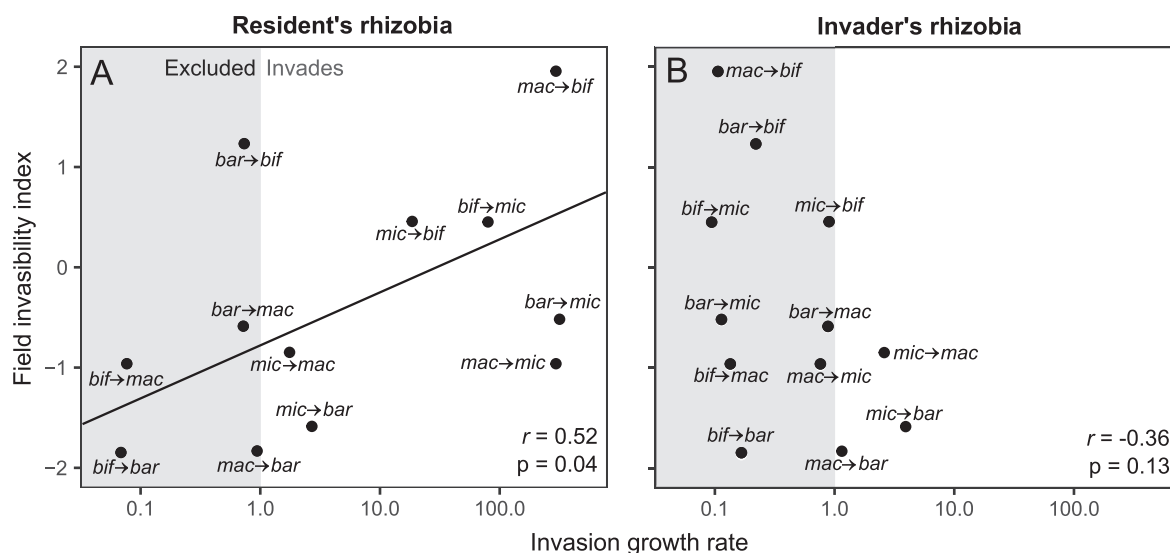


Figure 5: Rhizobial identity matters when linking predicted *Trifolium* species coexistence to field dynamics. Depicted are relationships between field coexistence dynamics and greenhouse-estimated invasion growth rates for each focal species invading each congener in the presence of rhizobia isolated from the congener (A) or from the focal species (B). Field invasibility index was measured as the effect of the presence of a species in the previous year on the presence of the focal species in the next year using data from five hundred sixty-three 0.09-m² plots at Bodega Marine Reserve, Bodega Bay, California, surveyed in April 2015 and 2016. Invasion growth rates were estimated using competition models parameterized from a greenhouse competition experiment. Invasion growth rates are shown on a log₁₀ scale for clarity. P values were obtained using permutation tests. bar = *T. barbigerum*; bif = *T. bifidum*; mac = *T. macraei*; mic = *T. microdon*.

of species to increase when rare (Chesson 2000b; Adler et al. 2007). Coexistence requires that each species in the community experiences negative frequency-dependent fitness, a positive per capita growth when rare, but then limits its own population growth most strongly when common. Although typically framed in the context of competition for abiotic resources, negative frequency dependence can also result from PSFs, for example, interactions with pathogens that build up in the environment when species become common (Janzen 1970; Connell 1971; Mills and Bever 1998; Van der Heijden et al. 2008). Our study adds strong support to the many studies that provide indirect evidence that PSFs mediated by soil pathogens (Packer and Clay 2000; Kulmatiski et al. 2008; Mangan et al. 2010; Reinhart 2012; Heinze et al. 2015) can stabilize coexistence by decreasing the relative performance of species in the presence of their own microbes.

PSF and Coexistence

Several studies have found that negative PSFs caused by soil pathogens are stronger when plants are grown in interspecific competition than in intraspecific competition or alone (Van der Putten and Peters 1997; Kardol et al. 2007; Petermann et al. 2010; Shannon et al. 2012; Pendergast et al. 2013), while other studies have found that negative PSFs were reduced (Casper and Castelli 2007) or unaffected (Maron et al. 2016) by the presence of heterospecific competitors. In our study, PSFs mediated by mutualistic rhizobia enhanced the ability of some *Trifolium* species to increase when rare, one key aspect of stable coexistence. Previous studies have shown that soil microbes and associated PSFs can influence plant interactions (Hartnett et al. 1993; Van der Putten and Peters 1997; Wagg et al. 2011; Lin et al. 2015; Bennett and Cahill 2016), in some cases altering the relative strength of intraspecific versus interspecific competition and increasing self-limitation (Pendergast et al. 2013; Chung and Rudgers 2016). By experimentally examining effects of PSFs on negative frequency dependence, relative fitness, and stabilizing niche differences, this study provides a direct test of a key mechanism hypothesized to maintain plant species diversity.

Our study advances on previous work by determining how interactions between *Trifolium* and rhizobia associated with conspecifics versus congeners contribute to relative fitness differences and stabilization, which collectively drive coexistence (or competitive exclusion). Interestingly, although we found a mix of negative, positive, and neutral frequency dependence among focal species in mixed *Trifolium* communities (fig. 1), frequency dependence became generally more negative in the presence of congeneric rhizobia when explored in a pairwise framework. We found that three of the four focal *Trifolium* species (all but *T. bifidum*) performed

best at low frequency and in the presence of congeneric rhizobia (figs. 2, 3), the conditions they would face as invaders into a community of congeners. This result suggests that rhizobial PSFs had a generally stabilizing effect that prevents, or at least slows, the rate of competitive exclusion. The feedbacks we measured were not strictly pairwise, since congener-origin rhizobia were sourced from three species, but they were realistic—68% of our small quadrats were occupied by more than one *Trifolium* species.

Rhizobia Mediate Coexistence through Both Stabilizing Niche Differences and Relative Fitness

Understanding the effects of a given mechanism on coexistence is complicated by the fact that it can influence both relative fitness differences and stabilizing niche differences. Our results demonstrate that relative fitness differences and stabilization depend on the rhizobia with which *Trifolium* associate (fig. 4A vs. 4B), with important implications for how we interpret these PSFs to influence coexistence. We assume that invasion growth rates when rare (i.e., coexistence) are best predicted by relative fitness differences and stabilization when the invading species associates with the resident species' rhizobia (i.e., congeneric rhizobia; fig. 4A). We suggest that the comparison of relative fitness differences and stabilization with congeneric vs. conspecific rhizobia (fig. 4A vs. fig. 4B) helps illustrate the role of congeneric rhizobia for coexistence. When invading *Trifolium* species were associated with congeneric rhizobial strains, the relative fitness difference between the resident (i.e., congener) and invader tended to increase (fig. 4A vs. 4B). In the absence of stabilizing effects, this feedback would lead to the competitive exclusion of the invading species, preventing coexistence. However, the increase in relative fitness differences when invading species associated with congeneric rhizobia was overcome by an even greater increase in stabilizing effects for most species pairs. These stabilizing effects, arising from strong competitive self-limitation of resident species in the presence of conspecific rhizobia, allow invading species to do better when they are rare, thus increasing the likelihood of coexistence. Whether enhanced self-limitation is a common outcome of PSFs remains unclear, since few feedback studies have directly addressed this question (but see Chung and Rudgers 2016). Understanding the mechanisms by which PSFs alter the relative strength of interspecific and intraspecific competition should be a focus of future coexistence research, which could integrate transcriptomic analysis with competition experiments (e.g., Bowsher et al. 2017).

We did not explore how *Trifolium* communities influence rhizobial community composition and over what time-scales. However, given that these are annual plants we know that they form nodules early in life and sustain those interactions through flowering; we thus anticipate that most

shifts in rhizobium communities that impact fitness occur between seasons rather than within a season, since at the end of the season nodules senesce and rhizobia are released in high numbers into the soil. Our study examined frequency dependence, stabilization, and relative fitness of *Trifolium* in rhizobial treatments that represent two snapshots of what focal *Trifolium* species would experience when going from rare (i.e., all congeneric) to abundant (i.e., all conspecific) in the community. Within- and between-season dynamics not captured by these metrics are relevant to the coexistence dynamics within both *Trifolium* and *Rhizobium* communities and are the subject of ongoing work.

Combining Greenhouse Experiments and Field Observations

An important contribution of our study is linking invasion dynamics and coexistence components as estimated in our greenhouse experiment to observed patterns of species coexistence in natural *Trifolium* communities. Coupling long-term observational records of field coexistence at fine spatial scales with greenhouse experimental results showed that our simplified communities could explain significant aspects of coexistence in biologically complex communities. PSF studies have linked single measures of field abundance with greenhouse-measured feedback; long-term field studies add an important temporal component to feedback studies, as feedbacks predict changing relative abundances through time. More studies combining these two types of data sets will, we feel, provide additional insights into the mechanisms underlying species coexistence. When we parsed invasion growth rates into relative fitness differences and stabilizing niche differences, we found that both were positively correlated with field coexistence, but neither alone was statistically significant ($P = .08$ and 0.15 , respectively; fig. 5); rather, both processes together contributed to the significant relationship with invasion growth rates.

Limitations of Our Study

Our study had two significant limitations. We did not manipulate rhizobial frequencies within communities, and our results suggest that these could play into the dynamics of *Trifolium* coexistence. A greater understanding of the rhizobia and their effects in combination and at different densities is the focus of ongoing work. The second key limitation in our modeling is that we did not include a seed bank phase, which can play a large role in stabilizing annual plant coexistence through the storage effect (Chesson 2000a, 2000b). In our system, we lack knowledge of differential seed dormancy and survival. Some species of *Trifolium* have seed dormancy of more than 90 years, and other legumes have been shown to have dormancy of 10,000 years in the arctic (Rice

1989). While future efforts will generate data on seed survival and dormancy, we are encouraged by our ability to predict field-observed transitions in *Trifolium* from our rhizobial strain data, even without accounting for these effects.

Conclusion

Our results suggest that shared mutualists play a role in stabilizing the coexistence of species, even that of close relatives that share many other aspects of their niche (Weber and Strauss 2016). Mutualisms may thus be an underappreciated mechanism of the maintenance of plant diversity (Bever 1999; Chung and Rudgers 2016). We expect that variation in the outcome of interactions as a function of frequency is common and that complex coexistence dynamics might occur in other interactions, such as plant associations with arbuscular mycorrhizal fungi or pollinators (e.g., Schmid et al. 2016; Guitian et al. 2017). Our work suggests that feedbacks mediated by rhizobial mutualists promoted coexistence by increasing the strength of stabilizing effects, and these effects were consistent with species coexistence dynamics observed in natural *Trifolium* assemblages. Our study demonstrates that experimentally manipulating particular interactions and directly quantifying their effects on equalizing and stabilizing mechanisms can provide valuable insights into how species coexist.

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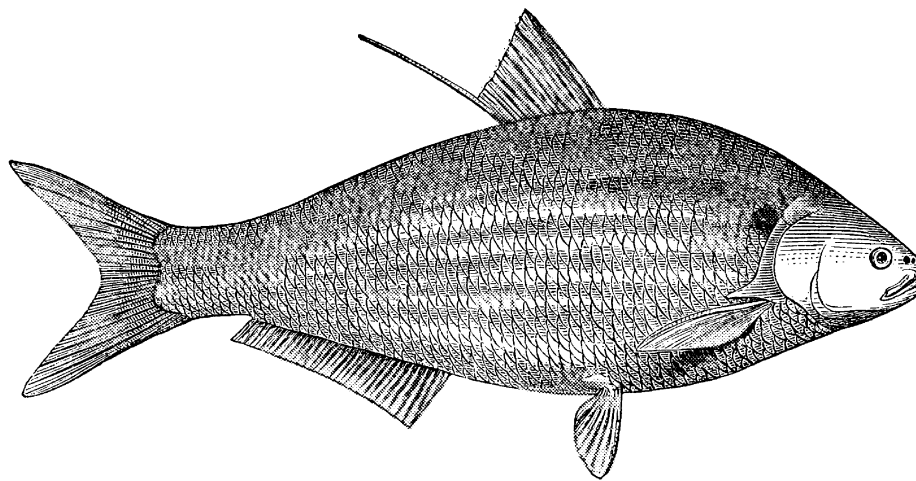
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“Besides this pond spoken of we know of one or two creeks that are annually visited by a few of these herring, and have occasionally seen several bushels hauled up from the deep holes in the creeks they had entered.” Figured: “Gizzard Shad, *Dorosoma Cepedianum*.” From “Notes on Fresh-Water Fishes of New Jersey” by Charles C. Abbott (*The American Naturalist*, 1870, 4:99–117).