

Decreased coevolutionary potential and increased symbiont fecundity during the biological invasion of a legume-rhizobium mutualism

Camille E. Wendlandt,¹ Emily Helliwell,¹ Miles Roberts,¹ Kyle T. Nguyen,¹ Maren L. Friesen,² Eric von Wettberg,³ Paul Price,⁴ Joel S. Griffitts,⁵ and Stephanie S. Porter^{1,6}

¹School of Biological Sciences, Washington State University, Vancouver, Washington

²Department of Plant Pathology, Department of Crop and Soil Sciences, Washington State University, Pullman, Washington

³Department of Plant and Soil Science, Gund Institute for the Environment, University of Vermont, Burlington, Vermont

⁴Department of Biology, Eastern Michigan University, Ypsilanti, Michigan

⁵Department of Microbiology and Molecular Biology, Brigham Young University, Provo, Utah

⁶E-mail: stephanie.porter@wsu.edu

Received June 12, 2020

Accepted December 7, 2020

Although most invasive species engage in mutualism, we know little about how mutualism evolves as partners colonize novel environments. Selection on cooperation and standing genetic variation for mutualism traits may differ between a mutualism's invaded and native ranges, which could alter cooperation and coevolutionary dynamics. To test for such differences, we compare mutualism traits between invaded- and native-range host-symbiont genotype combinations of the weedy legume, *Medicago polymorpha*, and its nitrogen-fixing rhizobium symbiont, *Ensifer medicae*, which have coinvasion North America. We find that mutualism benefits for plants are indistinguishable between invaded- and native-range symbioses. However, rhizobia gain greater fitness from invaded-range mutualisms than from native-range mutualisms, and this enhancement of symbiont fecundity could increase the mutualism's spread by increasing symbiont availability during plant colonization. Furthermore, mutualism traits in invaded-range symbioses show lower genetic variance and a simpler partitioning of genetic variance between host and symbiont sources, compared to native-range symbioses. This suggests that biological invasion has reduced mutualists' potential to respond to coevolutionary selection. Additionally, rhizobia bearing a locus (*hrrP*) that can enhance symbiotic fitness have more exploitative phenotypes in invaded-range than in native-range symbioses. These findings highlight the impacts of biological invasion on the evolution of mutualistic interactions.

KEY WORDS: Biological invasion, coevolution, *hrrP*, legume, mutualism, rhizobium.

Ecological communities are increasingly shaped by biological invasions of non-native species, which can reduce biodiversity and decrease the quality of ecosystem services (Ferlian et al. 2018; Guy-Haim et al. 2018; Vanbergen et al. 2018; Castro-Diez et al. 2019). Although invading species can rapidly evolve adaptations

to novel environmental conditions (Ochocki and Miller 2017; Getman-Pickering et al. 2018; Owen and Lahti 2020), they can also evolve altered species interactions (Yoshida et al. 2007), as is well established for antagonistic interactions (Carroll 2007; Osnas et al. 2015; Feis et al. 2016; Irimia et al. 2019; Pankoke

et al. 2019; Li et al. 2020). Despite the fact that most invasive species engage in mutualism, we know little about how invasion affects the evolution of positive species interactions (Richardson et al. 2000; Le Roux et al. 2017). Coevolution between mutualist species ultimately depends on the presence of partners' reciprocal fitness effects as well as genetic variance in these fitness effects (Gomulkiewicz et al. 2007), both of which can change over the course of invasion.

The levels of cooperation between mutualists may evolve rapidly if selection on mutualism changes during a biological invasion. On one hand, selection could shift to favor enhanced cooperation in a species' invasive range, if mutualistic interactions give the invader a fitness advantage over less cooperative conspecifics or native species in the recipient community (Rodríguez-Echeverría et al. 2008; Rodríguez-Echeverría et al. 2012; but see Bunn et al. 2015). On the other hand, selection could shift to favor reduced cooperation in a species' invasive range (Shelby et al. 2016; terHorst et al. 2018), if mutualism traits trade-off with traits that enhance colonization ability, like allocation to reproduction (Seifert et al. 2009). Consistent with this idea, legume species that do not associate with rhizobia are more successful invaders than legume species that do associate with rhizobia (Simonsen et al. 2017).

Mutualism evolution manifests as changes to mutualism traits. We define mutualism traits as traits expressed in the presence of interacting partners (i.e. joint phenotypes; Queller 2014) and/or traits that impact the resources or services exchanged in mutualism. Mutualism traits, thus, include cooperation outcomes, such as the degree of fitness benefit a partner obtains, attributes of symbiotic structures, like legume root nodules or squid light organs, and traits that can be measured on noninteracting partners, such as shoot mass, which affects a plant's ability to provision microbial symbionts with photosynthetically derived carbon, or proboscis length in insect pollinators, which affects the efficiency of pollination. Although studies have compared mutualism traits of invaded- and native-range genotypes during interactions with a common partner genotype (Seifert et al. 2009; terHorst et al. 2018), comparing the mutualism traits of invasive and native conspecifics with their respective regionally available mutualist pools (Shelby et al. 2016) more directly compares mutualism traits between ranges, since these are the genotypes actually interacting in nature.

The evolution of cooperation between mutualists depends on genetic variation in mutualism traits, which could decrease or increase during invasion. Changes in the magnitude of mutualism trait variation could alter the coevolutionary potential of mutualist species because genetic variance in mutualism traits underlies the potential of mutualist species to coevolve (Gomulkiewicz et al. 2007). Joint phenotypes evolve as the sum of additive genetic variance in each partner species, weighted by the selection

on each species (Queller 2014). However, the effects of invasion on quantitative variation for mutualism traits are not well understood. Stochastic colonization processes and genetic bottlenecks often reduce quantitative genetic variation in invading populations relative to native-range populations (Dlugosch and Parker 2008; Bacigalupe et al. 2013) which can constrain evolution in the invaded range (Colautti and Barrett 2011) and could reduce the ability of invaders to coevolve with mutualists. Alternatively, admixture among diverse source populations can increase quantitative genetic variation in invading populations relative to native source populations (Kolbe et al. 2008; Qiao et al. 2019; but see Chapple et al. 2013) and this could increase the potential for coevolution between mutualists in the invaded range. By assessing many host $\times$ symbiont genotypic combinations, it is possible to partition quantitative genetic variation for mutualism traits into amounts explained by the host genotype, the symbiont genotype, and the host $\times$ symbiont genotype interaction (Heath and Nuismer 2014) and to compare amounts of standing genetic variation in mutualism traits between the invaded and native ranges. This approach makes it possible to compare coevolutionary potential in the invaded and native range of a mutualism.

Legumes are frequent biological invaders around the globe (Richardson and Rejmánek 2011) and therefore the legume-rhizobium symbiosis provides an important model system which examines the impacts of invasion on the evolution of cooperation. Rhizobium soil bacteria infect the roots of legumes and are sequestered in host-derived intracellular symbiosomes within root nodules, where they can fix nitrogen and enhance the growth of their plant hosts (Oldroyd et al. 2011). Some legumes, including species in the medic clade, cause rhizobia within symbiosomes to terminally differentiate into highly efficient nitrogen fixation factories that can no longer reproduce outside the plant (Alunni and Gourion 2016). Nevertheless, a portion of the nodule's rhizobium population remains undifferentiated and can return to a free-living state in the soil following nodule senescence (Timmers et al. 2000; Oono et al. 2011). Legumes acquire diverse rhizobial symbionts from the environment rather than via vertical transmission from a parent, causing the fitness interests of rhizobia and plants to be imperfectly aligned (West et al. 2002). Selection can promote reduced cooperation in the mutualism, such as by favoring rhizobia that fix less nitrogen for their hosts (Porter and Simms 2014), or selection can promote higher cooperation in the mutualism such as in environments where nitrogen is scarce (Weese et al. 2015; Klinger et al. 2016). If greater investment in mutualism better enables partners to colonize novel habitats during biological invasions, selection could favor increased cooperation in the invaded range compared to the native range. On the other hand, if partners that invest less in cooperation and are less dependent on mutualism are better able to colonize novel

habitats, potentially due to low mutualist density in novel habitats, selection could favor reduced cooperation in the invaded range compared to the native range.

We rarely know the identity of genes that modulate the fitness benefits of mutualism for each partner (Baskett and Schemske 2015), but recently Price et al. (2015) identified one such locus in the rhizobium *Ensifer meliloti*. Here the plasmid-borne rhizobium *hrrP* gene encodes a metallopeptidase that allows rhizobia to degrade host-derived signals to terminally differentiate into nonreproductive, nitrogen-fixing cells (Price et al. 2015). Thus, rhizobia bearing the plasmid harboring *hrrP* may decrease nitrogen fixation and retain higher reproductive viability in the nodule than rhizobia lacking *hrrP* (Price et al. 2015), making *hrrP* a potentially key gene negotiating the fitness outcomes of this mutualism. Despite the large effect *hrrP* can have on cooperation (Price et al. 2015; Pan and Wang 2017), *hrrP* has yet to be studied in the context of wild populations of rhizobia and legume hosts. If selection favors increased cooperation during invasion, *hrrP* could be lost from rhizobium populations as they invade, and the exploitative effect of individual *hrrP* alleles could decrease. However, if selection favors reduced cooperation during invasion, *hrrP* could be selectively maintained, and the exploitative effect of *hrrP* alleles could increase.

Here, we study how levels of cooperation differ between the invaded and native ranges of a legume-rhizobium mutualism, focusing on the invasive weedy medic *Medicago polymorpha* and its symbiont *Ensifer medicae*. We compare genotypes sampled at the same time from the invaded and native ranges of these species, which is a common approach in invasion biology (Shelby et al. 2016; Lu-Irving et al. 2019; Pankoke et al. 2019). Our study assesses the net effect of many stages of biological invasion on symbiotic interactions, integrating the evolutionary changes that can occur throughout the invasion process (Zenni et al. 2017). Our approach does not determine whether differences between invaded- and native-range symbioses are due to effects of initial colonization (Brown et al. 2014) or due to subsequent years of evolution in separate ranges (Li et al. 2020). Genomics and historical records suggest that *M. polymorpha* and *E. medicae* were reintroduced to North America from the Mediterranean in the 1700s (Wing 1912; Porter et al. 2018). *Medicago polymorpha* has retained adaptive potential in the invaded range (Porter et al. 2011; Helliwell et al. 2018), even though it also experienced a genetic bottleneck that reduced its genetic diversity in the invaded range relative to the native range (Helliwell et al. 2018). In contrast to *M. polymorpha*, *E. medicae* did not experience a genetic bottleneck during invasion (Porter et al. 2018; but see Harrison et al. 2017b) and is less symbiotically specialized than its host (Bena et al. 2005), forming symbioses with several *Medicago* species that are also exotic in North America including the crop alfalfa (Garau et al. 2005; Denton et al. 2007). We leverage a ge-

ographically broad collection of invaded- and native-range genotypes of *M. polymorpha* and *E. medicae* to ask: (1) Has invasion affected mutualism fitness benefits for either mutualist partner? (2) Has invasion affected standing genetic variation in mutualism traits and how variance is partitioned between host and symbiont? (3) Has invasion affected the population-level frequency of *hrrP* or its exploitative phenotype?

Materials and Methods

ENSIFER STRAIN COLLECTION

We isolated a collection of *Ensifer* strains from field soils collected within wild *M. polymorpha* populations in the invaded (North American) and native (European) ranges of this species in 2014 and 2015 (Helliwell et al. 2018). We raised axenic *M. polymorpha* seedlings from each sampled population in a greenhouse in 2016 and inoculated plants with soils from their home population. We surface-sterilized root nodules from soil-inoculated plants and isolated one pure rhizobium culture (hereafter, “strain”) per nodule by culturing on tryptone-yeast (TY) media following Porter and Rice (2013). In total, we isolated 460 rhizobium strains from 36 *M. polymorpha* populations (Fig. 1), using 2–4 plant maternal lineages per population, 1–6 replicate plants per maternal lineage, and 1–6 nodules per plant. Each *M. polymorpha* population yielded 5–28 rhizobium strains. We consider rhizobia from this collection as presumptive *E. medicae*, since 16S sequencing of 158 *M. polymorpha* nodule isolates reveals that 97.5% are *E. medicae* (Porter and Rice 2013).

PCR SCREEN OF ENSIFER FOR *hrrP*

We assessed the prevalence of *hrrP* in the *E. medicae* strain collection using an established PCR screen (Price et al. 2015). Strains were cultured in 1 mL TY broth at 30°C and 280 rpm for 72 h, and genomic DNA was extracted from bacterial pellets using the Zymo gDNA minikit according to the manufacturer’s instructions. Approximately 10 ng/μL of gDNA was used as a template for PCR with *hrrP*-specific primers oPP017F and oPP020R (Price et al. 2015), yielding a 1.1 kb product. Thermocycler conditions were 98°C for 3 min, 35 cycles of 98°C for 15 s, 56°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products of the correct size were purified and Sanger sequenced. Strains were identified as *hrrP*⁺ if the PCR product had sequence homology to known *hrrP* loci (Price et al. 2015), assessed by phylogenetic analysis with known *hrrP* homologs (see below). Strains were identified as *hrrP*⁻, by the absence of a PCR band, or by the production of an off-target amplicon verified by sequencing. This approach is not known to be biased but could underestimate the number of *hrrP*⁺ strains in the collection, if false negatives are common. In total, we genotyped 400

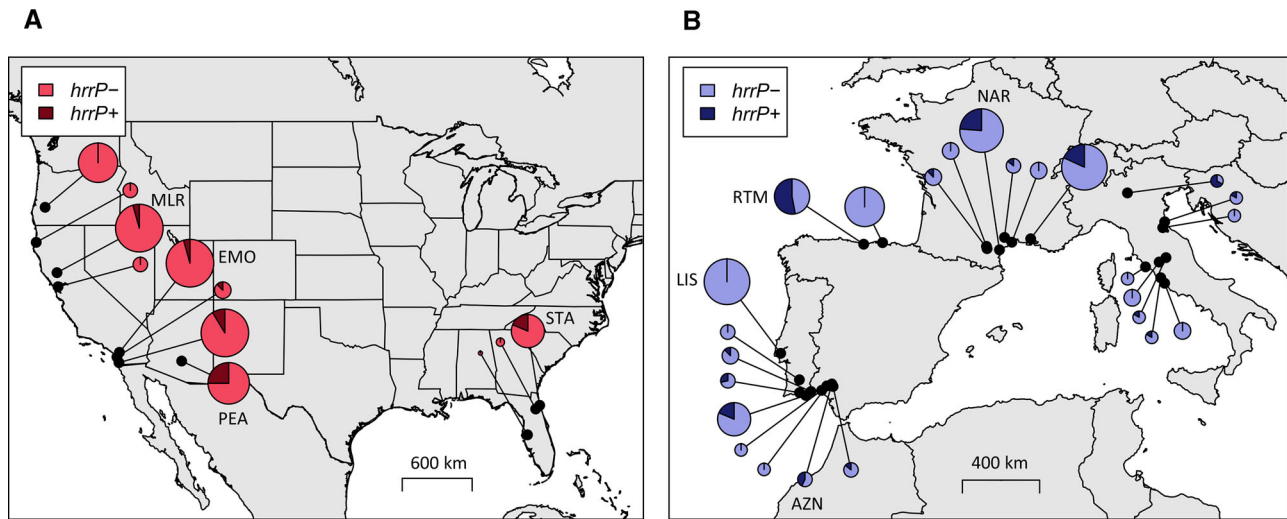


Figure 1. Sampled populations of *Medicago polymorpha* and *Ensifer medicae* show variable prevalence of the cooperation-modifying *E. medicae* locus, *hrrP*, in (A) the invaded-range North American populations and (B) the native-range European populations. Pie charts indicate frequency of *E. medicae* that test positive (dark) and negative (light) for the *hrrP* locus. Pie chart size is proportional to the number of *E. medicae* strains genotyped, which ranged from 2 to 23. The eight populations used in the greenhouse experiment are indicated with a three-letter code.

E. medicae strains from 36 *M. polymorpha* populations (152 strains from 11 invaded populations; 248 strains from 25 native populations; Supporting information Table S2). We estimated the frequency of *hrrP*+ strains in each population (“*hrrP* frequency”) as the number of *hrrP*+ strains divided by the number of strains genotyped in the population, which ranged from 2 to 23 strains; Supporting information Table S2).

PHYLOGENETIC ANALYSIS OF *hrrP*

To confirm that the PCR screen targeted *hrrP* homologs, we reconstructed a maximum likelihood phylogeny of 50 *hrrP* amplicon sequences from our study and 36 *hrrP* reference sequences from *Rhizobium* and *Ensifer*. Of the reference sequences, 27 were previously published (Price et al. 2015) and nine were amplified from additional isolates in the USDA *Ensifer* collection or obtained from Genbank. Our purpose was to assess whether *hrrP*-like sequences in our *E. medicae* population were phylogenetically intermingled with other *hrrP* homologs, similar to the approach by Price et al. (2015). We aligned sequences using MUSCLE in MEGA 7.0.14 (Kumar et al. 2016) and trimmed the alignment to a 200-nt stretch common to all sequences. The Jukes–Cantor nucleotide substitution model was selected from 88 candidate models using the AICc method in jModelTest version 2.1.6 (Darriba et al. 2012). We reconstructed an unrooted maximum-likelihood phylogenetic tree in RAxML version 8.2.12 (Stamatakis 2014), assessed node support by performing 1000 bootstrap replicates of the best tree, and collapsed poorly supported nodes (less than 70% bootstrap support) into polytomies.

GREENHOUSE EXPERIMENTAL DESIGN

We performed a greenhouse inoculation experiment at Washington State University Vancouver (45.7328054° N, 122.635967° W) in 2017. Axenic *M. polymorpha* seedlings were inoculated with single strains of *E. medicae*, and we measured mutualism traits of inoculated plants after five to seven weeks of growth. The experiment included 64 host × symbiont genotypic combinations (8 *M. polymorpha* genotypes × 8 *E. medicae* genotypes) from each range (invaded and native), replicated over six blocks, for a total of 768 inoculated plants. Each block also included two uninoculated control plants for each of the 16 *M. polymorpha* genotypes, for a total of 192 uninoculated plants. In total, the experiment included 960 plants arranged in a randomized complete block design. Plant and rhizobium genotypes were selected from collections of available genotypes to maximize variation for several parameters within each range (see Supporting information Table S1 and details below). Although we collected different numbers of *Ensifer* strains from the invaded and native ranges, and sampling depth varied among populations (Supporting information Table S2), the greenhouse experiment used the same strain and population sampling structure for each range, so the measurements of mutualism traits should be unbiased by range.

MEDICAGO GENOTYPES AND GROWTH CONDITIONS

The greenhouse experiment used 16 *M. polymorpha* genotypes from a collection of maternal lineages sampled from the same 36 wild populations that generated the *Ensifer* strain collection (Helliwell et al. 2018). We selected eight *M. polymorpha* populations (four populations per range) that had at least seven *E. medicae*

strains genotyped for *hrrP* and spanned the natural variation in *hrrP* frequency within each range (0–25% in the invaded range; 0–67% in the native range; Supporting information Table S2). We randomly chose two *M. polymorpha* genotypes from each selected population, for 16 plant genotypes total (Supporting information Table S1).

Seeds for the greenhouse experiment were generated in a greenhouse common garden to minimize maternal effects. *Medicago polymorpha* seeds were scarified on sandpaper on 6 July 2017, stratified at 4°C for six days, surface sterilized by exposure to chlorine gas for 6 h, and returned to cold storage for five days until sowing into autoclaved 158-mL containers filled with a 1:1 mix of Sungro Sunshine Mix #1 and sand. Plants were mist irrigated for 10 min twice daily and fertilized with 2 mL 0.5 X Fahreus solution (Vincent 1970) with 500 μ M NH_4NO_3 . Plants were placed in randomized block positions three days postinoculation, and pots were topped with autoclaved sand around each seedling to minimize cross-contamination. Fertilization began one week after inoculation with *E. medicae* and continued twice per week (six times total) until harvest.

ENSIFER GENOTYPES AND INOCULUM PREPARATION

The greenhouse experiment used 16 *E. medicae* strains from the eight populations chosen above, selecting one *hrrP*-strain from each population and two *hrrP*+ strains from each of the four populations with the highest *hrrP* frequency (Supporting information Table S1). For *hrrP*+ strains from the same population, we chose strains to maximize the genetic diversity sampled from the population. We confirmed the presence or absence of *hrrP* in each strain by PCR just prior to preparing inocula. To prepare *E. medicae* inocula, we inoculated 1 mL TY broth from frozen *E. medicae* stocks, incubated cultures at 30°C and 270 rpm for 48 h and used these to inoculate 24 mL TY broth cultures. After further incubation for 72 h, we centrifuged cells at 4000 rpm for 7 min and resuspended cell pellets in 0.1 X TY broth to a total concentration of 4×10^6 cells/mL, using the empirically derived formula, $\text{cells mL}^{-1} = \text{OD } 600 \times 5.8 \times 10^7$. We inoculated *M. polymorpha* with 900 μ L of *E. medicae* suspension (or a cell-free 0.1 X TY broth control) on 31 July 2017.

PLANT HARVEST

Thirty-six negative control plants were harvested early on 21 August 2017 to check for root nodules (i.e. contamination). None had formed nodules, and these plants were dropped from all analyses. The remaining plants were harvested between 4 September and 22 September 2017, proceeding block by block to avoid a treatment bias in time of harvest. Shoots were clipped and dried at 60°C. Roots were washed and stored on ice. We excised the largest, reddest nodule from each nodulated plant and stored nodules in 4°C water overnight before measuring cross-

sectional nodule size and the number of *E. medicae* colony-forming units (CFU) inside the nodule (see “Nodule Culturing” below). Roots were stored at –20°C and thawed to count nodules. Of the 768 plants inoculated with *E. medicae*, all but one plant formed root nodules. Of 192 negative control plants, 94% remained nodule free. Eleven contaminated control plants were dropped from all analyses.

NODULE CULTURING

Excised nodules were transferred to individual wells of a 96-well plate with a mesh bottom and removable mesh top. The plate was manually agitated in bleach (75 s) followed by three thorough rinses in sterile water. Surface-sterilized nodules were transferred to 100 μ L sterile water in 96-well plates. Plates were scanned (Epson Perfection V600 Photo scanner) to create digital images in which to measure cross-sectional nodule area (hereafter, “size of the largest nodule”). Nodules were transferred to individual 1.5 mL microcentrifuge tubes with 100 μ L TY broth, crushed with sterile pestles, and serially diluted. The final six nodule dilutions were drop plated (Vincent 1970) onto TY agar with at least three technical replicates, and colonies were counted after 3–4 days of incubation at 28°C. Each cultured nodule typically provided 18 estimates of the number of *E. medicae* CFU (6 nodule dilutions $\times$ 3 technical replicates). Colony counts were multiplied by their dilution factor and averaged within a nodule to yield a single estimate of rhizobium CFU per nodule. We only used colony counts of 1–50 and ignored assays in which bacterial growth was saturated (i.e. where individual colonies could not be distinguished). If all six dilutions of a technical replicate showed saturated growth, we assigned a count of one to the most dilute assay in which saturated growth occurred, since that dilution must have contained at least one CFU. This adjustment let us infer a conservative estimate of the number of CFU present and only affected 87 nodules out of 732 nodules for which we obtained CFU data.

MEASURING MUTUALISM TRAITS

We estimated early and late plant fitness by measuring the vegetative size of *M. polymorpha* at an early timepoint (trifoliate leaf count; three weeks postinoculation) and at harvest (dry shoot mass; five to seven weeks postinoculation), since plant size predicts reproductive output for the congener *Medicago truncatula* (Heath 2010). Although early and late fitness estimates may be correlated, these metrics can differ if, for example, juvenile and adult plants have different interactions with soil microbes (Dudenhöffer et al. 2018). We also calculated relative growth for each inoculated plant by dividing leaf count (or shoot mass) by the mean leaf count (or shoot mass) of uninoculated control plants of the same genotype from the same block. We estimated rhizobium fitness by measuring the number of *E. medicae* CFU in the plant’s

largest nodule, the size of the largest nodule on a plant, and the total number of root nodules formed on a plant. We log-transformed *E. medicae* CFU per nodule prior to analysis since the data varied over five orders of magnitude. Rapid doubling times of bacteria in favorable conditions can generate high skew in population sizes, so the log distribution of these data is biologically plausible. To assess the balance of mutualism fitness benefits between the plant host and rhizobium symbiont, we also analyzed dry shoot mass per individual nodule (i.e. shoot mass divided by nodule count).

STATISTICAL ANALYSES

We analyzed data from the greenhouse study and the *Ensifer* PCR screen using generalized linear mixed models (GLMM) implemented with lme4 version 1.1.21 (Bates et al. 2015) in R version 3.5.2 (R Core Team 2018). Gaussian error distributions were used for all responses except nodule count (negative binomial errors and log link) and *hrrP* incidence in the *E. medicae* strain collection (binomial errors and logit link). We checked the appropriateness of error distributions using simulated residuals in DHARMA version 0.2.4 (Hartig 2019) and assessed effects of interest with likelihood ratio tests.

To test how mutualism traits and effects of *hrrP* on mutualism traits differ between invaded- and native-range symbioses, we modeled mutualism traits of inoculated plants ($n = 768$) with fixed effects of *hrrP* presence/absence, range (invaded or native), the interaction of strain *hrrP* presence/absence and range, *hrrP* frequency of the plant's source population, and the sympatric status of the genotypic combination (one quarter of genotypic combinations were sympatric at the population level; the remainder were allopatric). We also ran GLMMs separately within each range, omitting range terms. As additional tests of whether invasion has affected plant growth responses to rhizobia, we modeled leaf count and shoot mass with fixed effects of range, inoculum (inoculated or uninoculated), and the interaction ($n = 906$ – 909), and we modeled plant relative growth with fixed effect of range ($n = 754$ – 759). All analyses also included random effects of plant genotype (G_{plant}), rhizobium genotype ($G_{\text{rhizobium}}$), the plant $\times$ rhizobium genotype interaction ($G_{\text{plant}} \times G_{\text{rhizobium}}$), and block.

We used random effects models to infer broad-sense genetic variation from total variation in mutualism traits. When genotype is included as a random effect in a statistical model, the covariance parameter estimate for the genotype term can be interpreted as the genetic variance of the trait (Lynch and Walsh 1998; Heath 2010; Simonsen and Stinchcombe 2014). To evaluate how the plant host and rhizobium symbiont contribute to genetic variance in mutualism traits, we modeled each mutualism trait with three random genotype terms (G_{plant} , $G_{\text{rhizobium}}$, $G_{\text{plant}} \times G_{\text{rhizobium}}$) and block, to account for phenotypic plasticity due to environmental variation in the greenhouse. Genetic variance of mutualism traits was assessed separately for invaded- and native-range symbioses

($n = 384$). We standardized the three genetic sources of variance by dividing them by the total variance of the mutualism trait being analyzed (i.e. the sum of model-explained variance and residual variance). For traits analyzed with non-Gaussian models (i.e. nodule count), genetic variance was standardized to total model-explained variance, since residual variance was not calculated. To compare genetic variance of mutualism traits between ranges, we obtained 95% confidence intervals for each standardized genetic variance estimate (Lavergne and Molofsky 2007) using the percentile method for 10,000 nonparametric bootstrap replicates of each model with boot version 1.3-23 (Canty and Ripley 2019).

To test if ranges differ in the frequency of *hrrP*+ *E. medicae*, we modeled the incidence of *hrrP* (presence or absence) in the *E. medicae* strain collection with a fixed effect of range and random effect of population. We also ran this GLMM using only strains from populations with both *hrrP*+ and *hrrP*- strains (i.e. “mixed” populations), which focused on potential differences in *hrrP* frequency distinct from presence-absence variation among populations.

Results

BENEFITS FROM MUTUALISM

We find that *M. polymorpha* genotypes obtain similar mutualism benefits from invaded- and native-range symbioses, whether measured by plant relative growth (leaf count: $\chi^2 = 0.0044$, $P = 0.95$; shoot mass: $\chi^2 = 0.087$, $P = 0.77$; Fig. 2A, B) or by the effect of inoculation on plant size (leaf count: $\chi^2 = 0.0016$, $P = 0.97$; shoot mass: $\chi^2 = 0.056$, $P = 0.81$; Fig. 2C, D). However, leaf count is 6% higher for invaded-range plant genotypes than native-range genotypes (Table 1 & Supporting information Figs. S1 and S2). In contrast, mutualism benefits for *E. medicae* are 296% higher for invaded-range than native-range symbioses, in terms of the number of rhizobium CFU per nodule (Table 1 & Fig. 3). Among invaded-range symbioses, the number of rhizobium CFU per nodule is 102% higher when the plant and rhizobium are sympatric (i.e. isolated from the same field population) rather than allopatric (Table 1 & Fig. 3). Values for other mutualism traits are similar between the invaded and native ranges, and similar between sympatric and allopatric pairings in each range (Table 1).

VARIANCE IN MUTUALISM TRAITS

Mutualism traits generally show simpler genetic variance partitioning for invaded-range symbioses than native-range symbioses (Table 2 & Fig. 4). Leaf count and shoot mass are shaped only by G_{plant} for invaded-range symbioses, whereas both G_{plant} and $G_{\text{rhizobium}}$ shape these fitness benefits for native-range symbioses

Table 1. Likelihood ratio test χ^2 statistics for fixed effects in GLMMs modeling mutualism traits of *Medicago polymorpha* and *Ensifer medicae*, both across ranges and separately within the invaded and native ranges. Effect of invasion is denoted by the Range term, which compares invaded and native ranges.

Trait	Range	Model terms				
		<i>hrrP</i>	<i>hrrP</i> frequency	Sympatry	Range	Range x <i>hrrP</i>
Leaf count	Across	5.27*	10.77**	0.67	6.41*	0.11
	Invaded	4.15*	5.93*	0.014	na	na
	Native	2.22	5.34*	0.87	na	na
Shoot mass	Across	3.47 [†]	5.57*	1.60	1.48	0.52
	Invaded	4.00*	3.06 [†]	1.24	Na	na
	Native	1.71	2.72 [†]	0.45	na	na
Shoot mass per nodule	Across	8.01**	2.24	0.042	0.20	0.54
	Invaded	6.77**	3.41 [†]	0.30	na	na
	Native	3.74 [†]	0.74	0.096	na	na
Nodule count	Across	5.03*	2.64	0.030	0.22	0.68
	Invaded	3.79 [†]	0.32	0.49	na	na
	Native	2.74 [†]	2.01	0.35	na	na
Nodule size	Across	0.0077	1.47	0.033	0.79	0.19
	Invaded	0.14	0.53	0.41	na	na
	Native	0.054	0.96	0.80	na	na
Log(Rhizobium CFU per nodule)	Across	0.69	0.28	3.56 [†]	5.70*	0.067
	Invaded	1.39	0.043	5.73*	na	na
	Native	0.10	0.36	0.063	na	na

[†]P < 0.10

*P < 0.05

**P < 0.01

***P < 0.001

"na" indicates that the model term was not tested.

Table 2. Likelihood ratio test χ^2 statistics for random effects in GLMMs modeling mutualism trait variance for contributions from *Medicago polymorpha* (G_{plant}) and *Ensifer medicae* (G_{rhizobia}), for the invaded and native ranges.

Trait	Range	Model terms		
		G_{plant}	G_{rhizobia}	$G_{\text{plant}} \times G_{\text{rhizobia}}$
Leaf count	Invaded	23.81***	3.11	~0
	Native	136.87***	13.57***	0.29
Shoot mass	Invaded	62.27***	~0	~0
	Native	193.51***	49.61***	3.07
Shoot mass per nodule	Invaded	23.93***	49.68***	~0
	Native	6.40*	133.12***	54.65***
Nodule count	Invaded	17.68***	82.38***	0.88
	Native	73.34***	575.65***	64.49***
Nodule size	Invaded	2.64	11.81***	~0
	Native	~0	21.63***	1.88
Log(Rhizobium CFU per nodule)	Invaded	2.09	20.67***	4.53*
	Native	10.37**	94.20***	5.00*

[†]P < 0.10

*P < 0.05

**P < 0.01

***P < 0.001

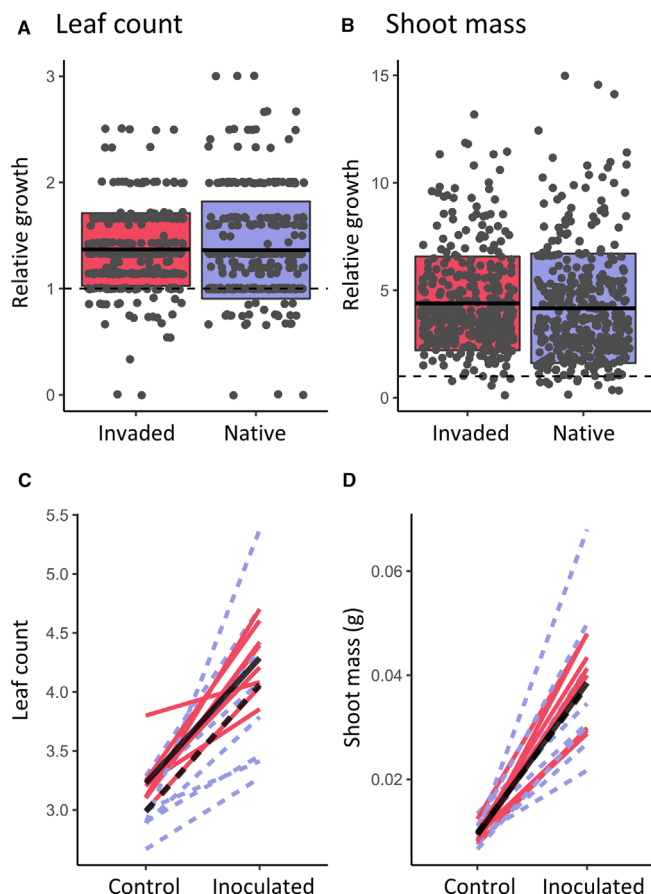


Figure 2. *Medicago polymorpha* benefits from *Ensifer medicae* in the native and invaded ranges are indistinguishable. Upper panels show plant relative growth calculated with (A) leaf count and (B) shoot mass. Values greater than 1 (dashed line) indicate a net increase in plant size in response to inoculation (relative growth = inoculated plant performance/uninoculated plant performance). Boxes indicate means $\pm$ SD. Each point is one replicate of $G_{\text{plant}} \times G_{\text{rhizobium}}$ combination. Points are jittered. Lower panels show plant size with and without rhizobia, for (C) leaf count and (D) shoot mass. Lines indicate mean change in plant size for one plant genotype in the uninoculated and inoculated treatments. Lines are colored by range (native range = blue dashed; invaded range = red solid). Thick black lines indicate mean response for each range (native range = dashed; invaded range = solid).

(Table 2). G_{plant} explains only 10% of the variance in leaf count for invaded-range symbioses, compared to 35% of the variance for native-range symbioses (Fig. 4A). Similarly, G_{plant} explains 19% of the variance in shoot mass for invaded-range symbioses, compared to 41% of the variance for native-range symbioses (Fig. 4B). Nodule count and shoot mass per nodule are shaped only by main genetic effects (G_{plant} and $G_{\text{rhizobium}}$) for invaded-range symbioses, whereas the $G_{\text{plant}} \times G_{\text{rhizobium}}$ interaction is also significant for native-range symbioses (Table 2 & Fig. 4C, D). Size of the largest nodule is shaped only by $G_{\text{rhizobium}}$, which

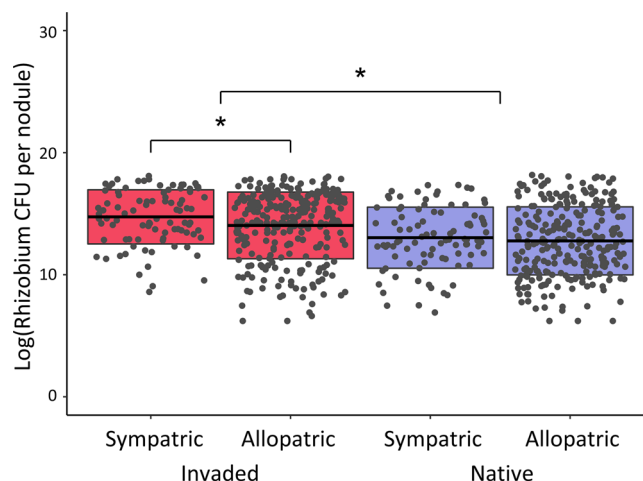


Figure 3. *Ensifer medicae* from the invaded range reap higher fitness benefits per nodule than *E. medicae* from the native range, particularly in symbiosis with sympatric *Medicago polymorpha*. Boxes indicate means $\pm$ 1 SD. Each point is one replicate of $G_{\text{plant}} \times G_{\text{rhizobium}}$ combination. Points are jittered. $*P < 0.05$.

explains similar amounts of variance for both invaded-range (7%) and native-range (11%) symbioses (Table 2 & Fig. 4E). Number of rhizobium CFU per nodule is shaped by $G_{\text{plant}} \times G_{\text{rhizobium}}$ for both invaded- and native-range symbioses (Table 2 & Fig. 4F).

POPULATION FREQUENCIES OF *hrrP*

The PCR screen identified *hrrP* in 50 of 400 *E. medicae* strains (12.5% of the collection). These putative *hrrP* sequences are nested among *hrrP* sequences from other rhizobium species, suggesting our PCR screen was successful at amplifying the target locus in *E. medicae* (Fig. 5). The incidence of *hrrP* is similar for invaded-range *E. medicae* (13/152 strains; 8.6% *hrrP*+) and native-range *E. medicae* (37/248 strains; 15% *hrrP*); χ^2 (1) = 1.21, $P = 0.27$, $n = 400$; Fig. 1), but there is significant variation in the incidence of *hrrP* among populations (χ^2 (1) = 14.78, $P = 0.00012$, $n = 400$). We identified *hrrP*+ *E. medicae* in 20/36 populations (56% of populations in the collection), with similar frequencies in the invaded range (6/11 populations; 55%) and native range (14/25 populations; 56%). Among strains from the 20 *E. medicae* populations where *hrrP* was detected (i.e., “mixed” populations), the incidence of *hrrP*+ strains is significantly lower in the invaded range (12%) than the native range (26%; χ^2 (1) = 5.28, $P = 0.022$, $n = 257$). However, permutation testing suggested the significant difference between ranges was probably an artifact of unequal sampling of populations from each range. We tested the effect of range in 1000 permuted datasets that maintained the population structure of our *Ensifer* collection (i.e. 6 invaded-range “mixed” populations and 14 native-range “mixed” populations). The 95% confidence interval for the likelihood ratio test statistic of range ($0.00073 < \chi^2 < 5.62$) encompassed

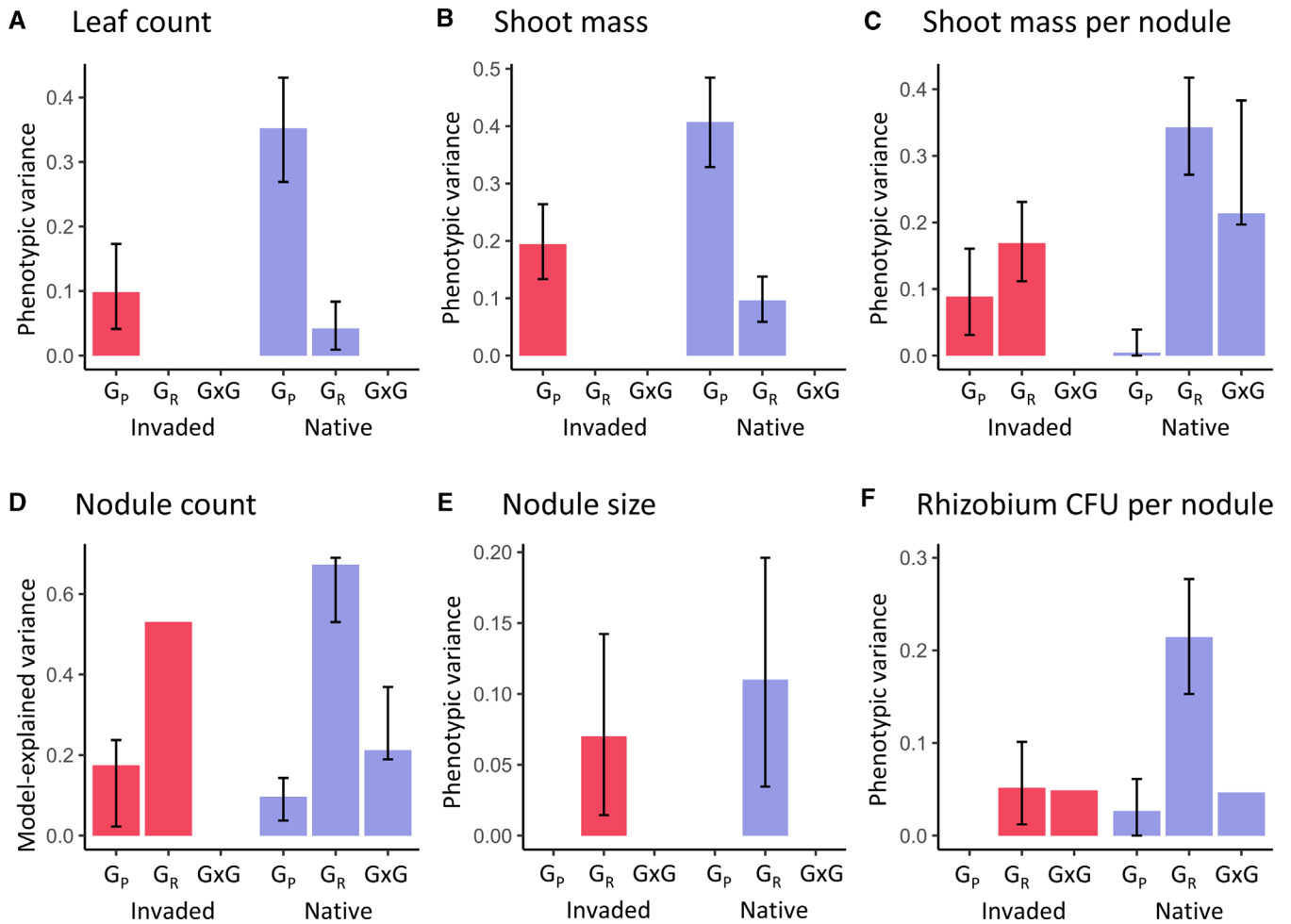


Figure 4. Invaded-range symbioses between *Medicago polymorpha* and *Ensifer medicae* have lower genetic variance in mutualism traits and simpler genetic variance partitioning compared to native-range symbioses. Mutualism trait variance is shown partitioned among *M. polymorpha* genotype (plant, G_P), *E. medicae* genotype (rhizobium, G_R), and the $G_{\text{plant}} \times G_{\text{rhizobium}}$ interaction ($G \times G$) for the invaded (red) and native (blue) ranges. Variance is expressed as the proportion of total phenotypic variance explained by G_P , G_R , or $G \times G$ for all traits except nodule count, which reports the total model-explained variance explained by the source. Only significant sources of variance are plotted. 95% confidence intervals for variance estimates were obtained with the percentile method on 10,000 nonparametric bootstraps of the model. Bars without confidence intervals indicate that the bootstrapped 95% confidence interval for the variance estimate did not include the original variance estimate; in these three cases (see panels D and F), we limit our interpretations to the presence or absence of a significant source of variance, rather than interpreting the magnitude of the variance.

our original test statistic ($\chi^2 = 5.28$), suggesting that the invaded and native ranges do not in fact differ in the incidence of *hrrP* in “mixed” populations.

EXPLOITATION ASSOCIATED WITH *hrrP*

Compared to *hrrP*- *E. medicae*, *hrrP*+ *E. medicae* confer lower mutualism benefits to *M. polymorpha* and obtain higher benefits for themselves, consistent with the *hrrP* locus being associated with rhizobium exploitation of plant hosts. Compared to plants in symbiosis with *hrrP*- *E. medicae*, plants in symbiosis with *hrrP*+ *E. medicae* have lower leaf count (6% lower across ranges; 5% lower in the invaded range), lower shoot mass (8%

lower in the invaded range; similar effect but only marginally significant across ranges), lower shoot mass per nodule (37% lower across ranges; 32% lower in the invaded range; similar effect but only marginally significant in the native range), and higher nodule count (63% higher across ranges; similar effect but only marginally significant within each range; Table 1 & Fig. 6). Across both ranges, measures of plant size are significantly associated with *hrrP* frequency of the plant’s source population: plants from the four populations with the highest *hrrP* frequency (24–53% *hrrP*) have 9% higher leaf count and 21% higher shoot mass than plants from the four populations with the lowest *hrrP* frequency (0–18% *hrrP*; Table 1 & Supporting information Fig. S3). The effect of local *hrrP* frequency on leaf count is also

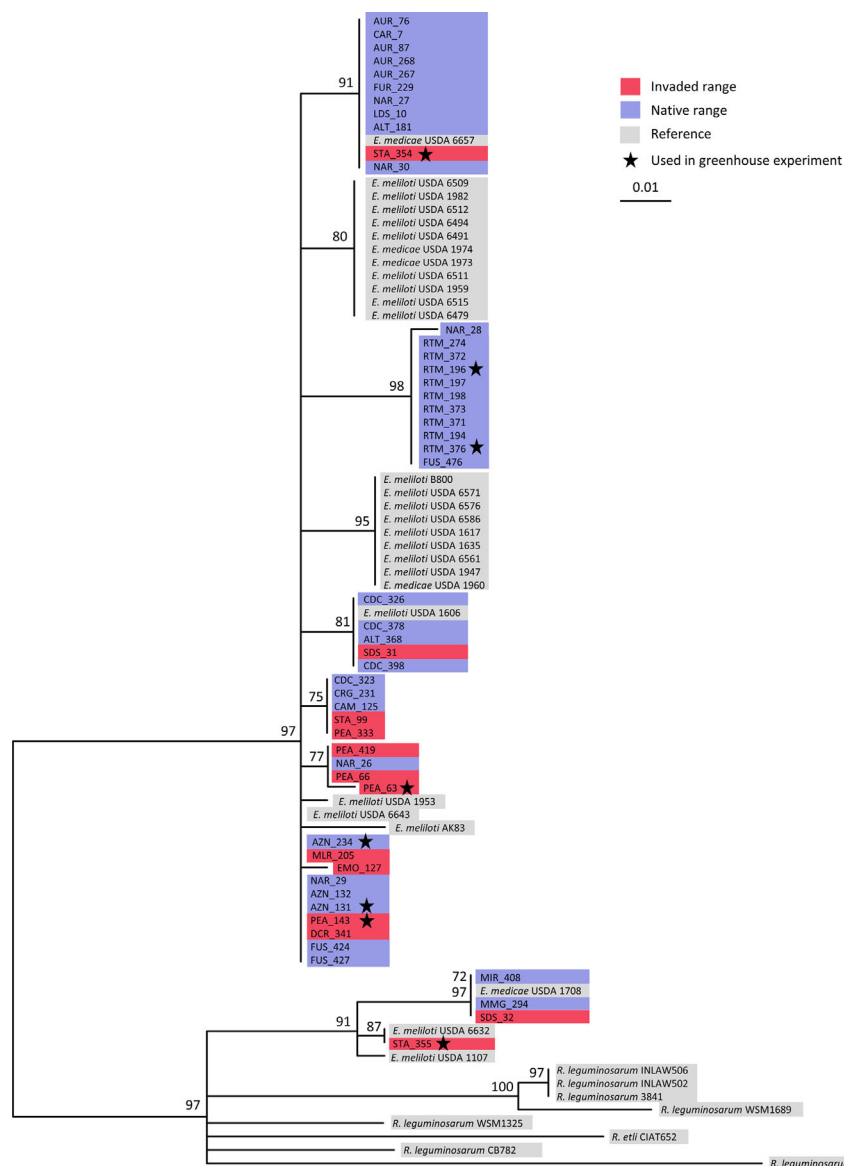


Figure 5. The 50 *hrrP* alleles sequenced from *Ensifer medicae* populations are nested within known diversity for *hrrP*. Midpoint-rooted maximum likelihood phylogenetic tree for 50 *hrrP* sequences from *E. medicae* (this study) and 36 *hrrP* orthologs from other *Ensifer* and *Rhizobium* species (indicated with the species name and USDA accession).

significant in separate analyses of invaded- and native-range symbioses (Table 1 & Supporting information Fig. S3).

Discussion

Despite the increase in human-mediated dispersal of mutualists, we are only beginning to understand how biological invasion alters cooperation between species (Seifert et al. 2009; Shelby et al. 2016; terHorst et al. 2018). Comparing genetic variation in traits of interacting species between their invaded and native ranges is a powerful approach for detecting shifts in cooperation due to invasion processes. To quantify how invasion has changed the ben-

efits of mutualism and genetic variance in mutualism traits for a coinvasive plant-microbe symbiosis, we examine 128 genotypic combinations of the legume *M. polymorpha* and its mutualistic rhizobium symbiont *E. medicae*. We find that plant hosts obtain similar mutualism benefits in both ranges, but symbionts obtain greater mutualism benefits from invaded-range symbioses than native-range symbioses. We also find that invasion has reduced standing genetic variation for several mutualism traits and simplified how variation is partitioned between partners, which could alter how mutualism traits respond to coevolutionary selection. Focusing on a symbiont locus, *hrrP*, which has the ability to underlie host-symbiont conflict (Price et al. 2015), we find that invaded and native ranges have similar population frequencies of

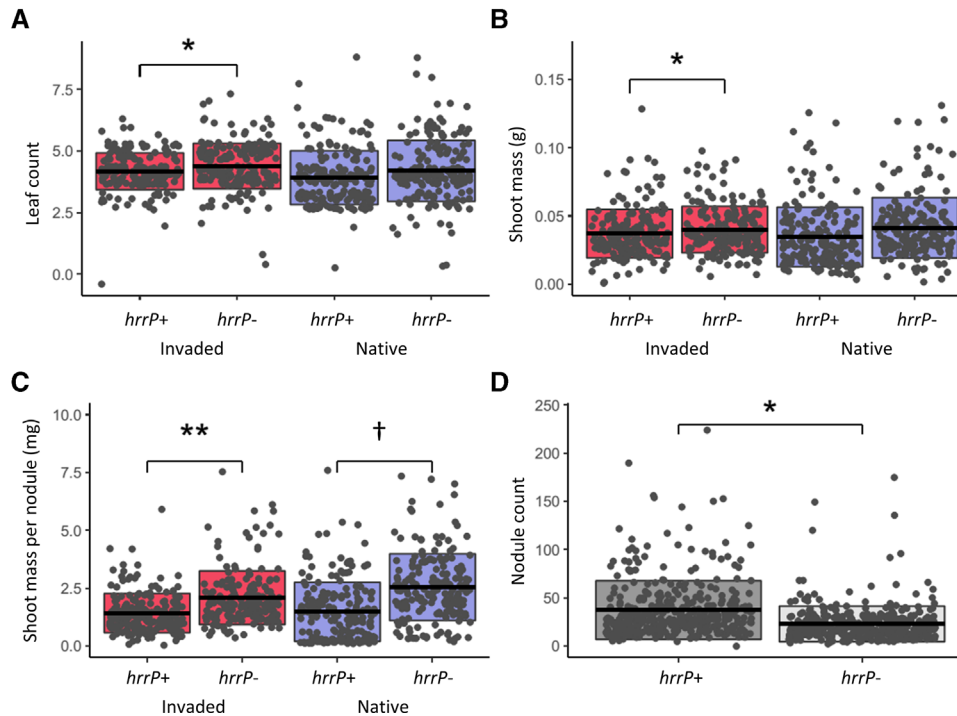


Figure 6. *Ensifer medicae* bearing *hrrP* reduce performance of *Medicago polymorpha* hosts and form more nodules than rhizobia lacking *hrrP*, especially in the invaded range. Only significant effects are shown; *hrrP* genotype had no effect on nodule size or rhizobium CFU per nodule. Boxes indicate means ± 1 SD. Each point is one replicate of $G_{\text{plant}} \times G_{\text{rhizobium}}$ combination. Points are jittered. * $P < 0.05$, ** $P < 0.01$, † $P < 0.10$.

hrrP, but higher levels of host exploitation associated with *hrrP* in the invaded range.

I. BENEFITS FROM MUTUALISM

The benefits of mutualism to *M. polymorpha* plants have been robustly maintained during its invasion of North America. This pattern is striking given that other studies show decreased mutualism benefits and reduced dependence on symbionts for invasive plants, potentially due to limited availability of mutualists in the introduced range (Shelby et al. 2016) or trade-offs imposed by shifting to a weedier life history (Seifert et al. 2009), which could reduce selection for traits that enable a host to benefit from mutualism. Previous work in this system shows that invaded-range symbionts are less beneficial to invaded-range *M. polymorpha* than to native-range *M. polymorpha* (terHorst et al. 2018). However, we find that plant genotypes from both ranges obtain similar benefits from symbiont genotypes within their region of origin. Our finding is consistent with a scenario in which any limitations to mutualist availability or tradeoffs *M. polymorpha* experiences in the invaded range have not imposed strong selection for reduced mutualism benefits or dependence on mutualists. Although it is possible that mutualism benefits have failed to respond to changes in selection on mutualism in the invaded range

due to insufficient genetic variation in *M. polymorpha*, this seems unlikely since *M. polymorpha* has undergone other evolutionary changes since invasion, including adaptive divergence in flowering time (Helliwell et al. 2018) and increased vegetative size (Getman-Pickering et al. 2018), a finding that we recapitulate.

The maintenance of mutualism fitness benefits likely facilitates the invasive success of *M. polymorpha*, since *M. polymorpha* lacking rhizobia have 75% lower shoot mass than inoculated plants. Coinvading symbionts also facilitate invasive *Acacia* on the Iberian Peninsula (Rodríguez-Echeverría et al. 2012), although facilitation in that system partly depends on coinvasive *Bradyrhizobium* disrupting symbioses of plants in the recipient community (i.e. symbiont spillover; Dickie et al. 2017). We have no evidence that *E. medicae* can nodulate native North American plants, suggesting that symbiosis facilitates invasion of *M. polymorpha* by directly increasing its fitness, rather than by weakening the biotic resistance of recipient communities.

Mutualistic microbes with access to alternative hosts can accelerate plant invasions, since these alternative hosts serve as reservoirs of compatible symbionts for the invader (Moeller et al. 2015). The maintenance of mutualism fitness benefits for invasive *M. polymorpha* may be due to high specificity for *E. medicae* (i.e. the plant host cannot obtain the mutualist service from another microbe), and high availability of *E. medicae*

in the new range, potentially due to codispersal or to *E. medicae* dispersing ahead of *M. polymorpha* by the use of alternative medic hosts such as alfalfa (Wing 1912; Porter et al. 2018) or *Medicago lupulina* (Harrison et al. 2017a). Coinvading plants and mutualistic microbes often continue associating with each other in their new range, even when they have access to novel mutualists, suggesting that codispersal is appreciable in nature (Bogar et al. 2015; Le Roux et al. 2017).

Mutualism fitness benefits are often more difficult to measure for microbes than for their plant hosts. To our knowledge, this is the first study to compare microbial symbiont fitness benefits from mutualism between invaded- and native-range host-symbiont pairings. Although the number and size of nodules formed in mutualism is indistinguishable between invaded- and native-range mutualists, *E. medicae* obtain greater numbers of CFU per nodule in invaded range mutualisms. The number of individuals that are introduced to found a population (propagule pressure) is a consistent determinant of the successful establishment of invasive species (Cassey et al. 2018; van Kleunen et al. 2018). Intriguingly, our finding that nodules harbor about 300% more reproductively viable rhizobia in symbioses from the invaded range compared to those from the native range suggests a mechanism for both partners' successful coinvasion and the maintenance of mutualism in the invaded range. By evolving to produce a greater number of reproductively viable rhizobia within a root nodule, symbioses between *M. polymorpha* and *E. medicae* are predicted to seed the soil with higher numbers of mutualistic rhizobia in the invaded range than in the native range. Host lineages could benefit from greater symbiont fecundity if greater symbiont propagule number feeds back to increase the fitness of a host lineage's progeny, possibly by alleviating any local shortage of symbiont propagules (Lopez et al. 2020; but see Wandrag et al. 2020). If selection favors lineages that increase environmental densities of symbiont propagules in novel, uncolonized areas, the evolution of higher symbiont fecundity could spur the success of mutualist coinvasions.

It is possible that novel or harsh selection regimes during the free-living life history phase within novel soil communities in the invaded range has increased the importance of symbiotic fitness for *E. medicae* and selected for strains that are able to produce more CFU per nodule. *Ensifer medicae* genomes from the invaded range are uniquely enriched in binding functions, relative to *E. medicae* genomes in the native range, which could indicate differences in selection on *E. medicae* between the ranges (Porter et al. 2018). Higher fitness benefits from cooperation could speed adaptation to novel environments if higher benefits from cooperation maintain higher population sizes, allowing a population to better adapt to novel environmental optima (Henriques and Osmond 2020).

We find that invasion has led to the evolution of an increase in benefits to *E. medicae* without altering mutualism benefits to *M. polymorpha*. Yet, in previous single strain inoculum experiments, *E. medicae* that reap higher fitness rewards from symbiosis confer lower benefits to *M. polymorpha* (Porter and Simms 2014). It is possible that preferential allocation of resources to more cooperative symbionts (Kiers and Denison 2008; Bever 2015) under conditions of natural coinfection has maintained plant benefits from the mutualism, despite the increased fitness rewards symbionts reap. Our assessment of mutualism fitness benefits in controlled greenhouse conditions does not account for ecological factors, such as symbiont population size and coinfection by multiple symbiont genotypes, and it would be useful for future studies to do so.

II. VARIANCE IN MUTUALISM TRAITS

We find that invasion has resulted in decreased genetic variance for mutualism traits and simplified how variance in these traits is partitioned between partners, which could alter how levels of cooperation respond to coevolutionary selection. By assessing the magnitude of plant and rhizobium genetic contributions to fitness benefits in each range, we identify ways that invasion alters coevolutionary options for these mutualists. In the native range of *M. polymorpha* and *E. medicae*, we find that early and late plant fitness estimates (leaf count and shoot mass, respectively) are determined in part by rhizobium genetic variance, but the invaded range lacks a rhizobium genetic contribution to plant fitness. Reduced variation in rhizobium mutualist quality in the invaded range could occur due to genetic bottlenecks, like the one experienced by *M. polymorpha* (Helliwell et al. 2018). However, similar genome-wide rates of diversity in the invaded and native range of *E. medicae* suggest this rhizobium species has not experienced a genomic bottleneck during invasion (Porter et al. 2018). It is also possible that increased stabilizing selection on rhizobium cooperation in the invaded range could have reduced the proportion of strains with high and low cooperation abilities, without altering mean rhizobium cooperation. Exotic genotypic combinations of plants and arbuscular mycorrhizal fungi tend to show less variance in plant responses than native genotypic combinations (Klironomos 2003), suggesting that loss of trait variance during host-symbiont invasions could be common. Whether reduced variation in *E. medicae* cooperation is due to genetic bottlenecks or stabilizing selection could be distinguished in future studies by evaluating signatures of selection on genes underlying mutualism traits in *E. medicae*, as has been done previously for medics (Bonhomme et al. 2015). Overall, our results show that biological invasion can eliminate entire sources of genetic variation in joint phenotypes (Queller 2014), causing fitness benefits

that are determined by the genotype of both partners in the native range to be determined by only one partner's genotype in the invaded range.

We also identify mutualism traits for which the contribution of a plant genotype to the trait value depends on the identity of the rhizobium genotype, and vice versa (i.e. a $G_{\text{plant}} \times G_{\text{rhizobium}}$ interaction) in the native range but not in the invaded range, suggesting a loss of coevolutionary potential during invasion (Heath and Nuismer 2014). In the native range, we detected $G_{\text{plant}} \times G_{\text{rhizobium}}$ variance for nodule count and shoot mass per nodule, suggesting that high values of these two traits require particular plant-rhizobium genotypic combinations, rather than simple main effects of plant or rhizobium genotype. However, we do not detect $G_{\text{plant}} \times G_{\text{rhizobium}}$ variation for nodule count or shoot mass per nodule in invaded range symbioses. Thus, nodule count and shoot mass per nodule have the potential to underlie *M. polymorpha*-*E. medicae* coevolution in the native range, but not the invaded range. The loss of $G_{\text{plant}} \times G_{\text{rhizobium}}$ variation in the invaded range suggests that the scope for coevolutionary selection has been reduced during invasion, making cooperation less evolvable in response to coevolutionary selection in the invaded range relative to the native range. Shoot mass per nodule may be an especially important trait to be underlain by $G_{\text{plant}} \times G_{\text{rhizobium}}$ variance because it reflects the balance of mutualism benefits to each partner. Hosts and symbionts can be in conflict over the value of this trait (Porter and Simms 2014; but see Friesen 2012), since the number and size of nodules formed represents an allocation of resources to symbiont fitness instead of plant fitness. Our findings are consistent with an overall pattern whereby $G_{\text{plant}} \times G_{\text{rhizobium}}$ interactions underlie many joint phenotypes in native range medic-*Ensifer* mutualisms (Heath 2010; Heath et al. 2012), but underlie fewer joint phenotypes in invaded range mutualisms (Porter and Simms 2014; Harrison et al. 2017a). A simplification of the genetic architecture of joint phenotypes due to the reduction of genotype $\times$ genotype interactions could be a common outcome of invasion processes if genetic variation for traits tends to be lost during the colonization of novel habitats.

The number of rhizobium CFU per nodule shows significant $G_{\text{plant}} \times G_{\text{rhizobium}}$ variance in both the invaded and native ranges, suggesting that this component of rhizobium fitness has retained coevolutionary potential during invasion. Furthermore, rhizobium CFU per nodule is also higher with sympatric than allopatric partners in the invaded range, which could be evidence of coevolution in the invaded range. However, since no plant fitness components vary with host-symbiont sympatry in our study, the evidence of coevolution between partners in the invaded range is weak. Instead of coevolution between partner species, it is possible that selection in the invaded range has favored rhizobia that specialize on local plant genotypes. *Medicago polymorpha* populations are less genetically diverse in the invaded range compared

to the native range (Helliwell et al. 2018) and lower plant diversity might enable symbionts to more rapidly adapt to their host populations to optimize symbiotic fitness. However, for weeds like *M. polymorpha* that are capable of rapid dispersal and colonization, homogenizing gene flow could act against selection for local adaptation, as seen with *M. lupulina* in eastern North America (Harrison et al. 2017a, b). Indeed, *M. polymorpha* shows low spatial genetic structure in the invaded range compared to the native range (Helliwell et al. 2018), consistent with low potential for coevolutionary dynamics with *E. medicae* in the invaded range.

III. EXPLOITATION IN MUTUALISM

We show that the *Ensifer* locus *hrrP* is associated with exploitation of legume hosts by rhizobium symbionts in this collection of wild *M. polymorpha* and *E. medicae* genotypes. Rhizobia with *hrrP* obtain greater mutualism fitness benefits and are less cooperative to plant hosts than rhizobia without *hrrP*. By degrading host cues for rhizobia to terminally differentiate, *HrrP* can allow rhizobia to maintain viability inside nodules and avoid allocating resources to the costly service of nitrogen fixation (Price et al. 2015; Pan and Wang 2017). The negative effects of *hrrP* on shoot mass per nodule particularly highlights how *hrrP* can shift the balance between plant and rhizobium benefits in this mutualism to favor rhizobia. Interestingly, plant lineages collected from populations with higher frequencies of *hrrP* in *E. medicae* are larger, tending to have higher genotypic values for leaf count and shoot mass. It is possible that frequent encounters between *M. polymorpha* and *hrrP*+ *E. medicae* in these populations select for larger plant size if increased vigor compensates for more exploitative rhizobia.

We find that *M. polymorpha* has invaded North America despite maintenance of the antagonism associated with *hrrP* in its partner *E. medicae*, with this locus becoming neither less frequent nor less antagonistic during invasion. The maintenance of this antagonistic locus without any decrease in overall levels of cooperation by rhizobia emphasizes that other loci contribute to variation in cooperation in rhizobia. One caveat is that our study cannot separate the effects of the *hrrP* locus from the effects of the plasmid bearing *hrrP*, which contains hundreds of other putative genes (Crook et al. 2012; Price et al. 2015). Future work would be necessary to isolate the specific effects of *hrrP*.

Overall, our study suggests that mutualistic microbes may act like facilitator species during ecological succession, promoting the establishment of their hosts while maintaining separate fitness interests that maintain selection for antagonistic loci. Although holobiont theory has sometimes been used to characterize plant-microbe interactions (Hassani et al. 2018), the mismatches we detect between host and symbiont fitness interests suggest that individual populations of hosts and symbionts are more

appropriate units of selection in this system (Koskella and Bergelson 2020).

Conclusions

The evolution of species interactions increasingly takes place in a human-influenced landscape characterized by biological invasions. Knowledge of how cooperation between mutualists is impacted by invasion has lagged behind knowledge of other evolutionary responses to invasion, despite the fact that most invasive species engage in mutualism. We help fill this knowledge gap by studying changes in the mean and variance of mutualism fitness benefits in an invasive legume-rhizobium system. By leveraging a geographically broad set of plant and rhizobium genotypes, we show that symbiosis can evolve to confer greater symbiont fecundity in the invaded range, which could spur increased colonization success by the symbiosis in novel habitats. We also show that invasions can reduce and simplify genetic variation in mutualism traits. Since genetic variance, and particularly $G \times G$ genetic variance, is required for response to coevolutionary selection, our work suggests that biological invasions can erode coevolutionary potential between mutualist species. Nevertheless, we also find that genotypes from both ranges have similar genetic potential for mutualism fitness benefits and similar levels of antagonism associated with the rhizobium locus, *hrrP*. Far from finding a mutualism in decline after invasion, we find a mutualism delivering robust benefits to each partner in both its old and new ranges, but with potentially less capacity respond to coevolutionary selection in the new range.

AUTHOR CONTRIBUTIONS

MLF and SSP generated the *E. medicae* strain collection. EH, PP, and JSG contributed to the PCR screen for *hrrP*. SSP, EH, and MLF designed the greenhouse experiment. EH, MR, and KTN performed the greenhouse experiment and collected data. CEW and SSP analyzed the data and wrote the manuscript. All authors contributed to editing.

ACKNOWLEDGMENTS

We thank the UC Natural Reserve System for permission to collect soil and seeds. We also thank Zoie Lopez and Christopher Dexheimer for assistance with seed amplification and data collection, Carissa Zielinski, and Emily McLachlan for assistance with strain isolations, and J. Pioviascott for statistical advice. We are grateful to the U.S. National Science Foundation (DEB-1355216 to S.S. Porter).

DATA ARCHIVING

Data and code are available at <https://doi.org/10.5061/dryad.3bk3j9khx>. *hrrP* DNA sequences from *Ensifer medicae* are available on Genbank (accessions MW417423-MW417472).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

LITERATURE CITED

- Alunni, B., and B. Gourion. 2016. Terminal bacteroid differentiation in the legume–rhizobium symbiosis: nodule-specific cysteine-rich peptides and beyond. *New Phytol.* 211:411–417.
- Bacigalupe, L. D., K. Barrientos, A. P. Beckerman, M. J. Carter, C. C. Figueroa, S. P. Foster, A. J. Moore, A. X. Silva, and R. F. Nespole. 2013. Can invasions occur without change? A comparison of G-matrices and selection in the peach-potato aphid, *Myzus persicae*. *Ecology and Evolution* 3:5109–5118.
- Baskett, C. A., and D. W. Schemske. 2015. Evolution and genetics of mutualism. *in* J. L. Bronstein, ed. *Mutualism*. Oxford Univ. Press, Oxford, U.K.
- Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67. <https://doi.org/10.18637/jss.v067.i01>
- Bena, G., A. Lyet, T. Huguet, and I. Olivieri. 2005. Medicago–Sinorhizobium symbiotic specificity evolution and the geographic expansion of Medicago. *J. Evol. Biol.* 18:1547–1558.
- Bever, J. D. 2015. Preferential allocation, physio-evolutionary feedbacks, and the stability and environmental patterns of mutualism between plants and their root symbionts. *New Phytol.* 205:1503–1514.
- Bogar, L. M., I. A. Dickie, P. G. Kennedy, and D. M. Richardson. 2015. Testing the co-invasion hypothesis: ectomycorrhizal fungal communities on *Alnus glutinosa* and *Salix fragilis* in New Zealand. *Diversity and Distributions* 21:268–278.
- Bonhomme, M., S. Boitard, H. San Clemente, B. Dumas, N. Young, and C. Jacquet. 2015. Genomic signature of selective sweeps illuminates adaptation of *Medicago truncatula* to root-associated microorganisms. *Mol. Biol. Evol.* 32:2097–2110.
- Brown, A. M. V., L. Y. Huynh, C. M. Bolender, K. G. Nelson, and J. P. McCutcheon. 2014. Population genomics of a symbiont in the early stages of a pest invasion. *Mol. Ecol.* 23:1516–1530.
- Bunn, R. A., P. W. Ramsey, Y. Lekberg, and M. van der Heijden. 2015. Do native and invasive plants differ in their interactions with arbuscular mycorrhizal fungi? A meta-analysis. *Journal of Ecology* 103:1547–1556.
- Canty, A., and B. D. Ripley. 2019. Boot: bootstrap R (S-Plus) function. Available at https://www.researchgate.net/publication/311467889_boot_Bootstrap_R_S-Plus_Functions
- Carroll, S. P. 2007. Natives adapting to invasive species: ecology, genes, and the sustainability of conservation. *Ecol. Res.* 22:892–901.
- Cassey, P., S. Delean, J. L. Lockwood, J. S. Sadowski, and T. M. Blackburn. 2018. Dissecting the null model for biological invasions: a meta-analysis of the propagule pressure effect. *PLoS Biol.* 16:e2005987.
- Castro-Diez, P., A. S. Vaz, J. S. Silva, M. van Loo, A. Alonso, C. Aponte, A. Bayon, P. J. Bellingham, M. C. Chiufo, N. DiManno, et al. 2019. Global effects of non-native tree species on multiple ecosystem services. *Biol. Rev. Camb. Philos. Soc.* 94:1477–1501.
- Chapple, D. G., K. A. Miller, F. Kraus, and M. B. Thompson. 2013. Divergent introduction histories among invasive populations of the delicate skink (*Lampropholis delicata*): has the importance of genetic admixture in the success of biological invasions been overemphasized? *Diversity and Distributions* 19:134–146.
- Colautti, R. I., and S. C. Barrett. 2011. Population divergence along lines of genetic variance and covariance in the invasive plant *Lythrum salicaria* in eastern North America. *Evolution* 65:2514–2529.
- Crook, M. B., D. P. Lindsay, M. B. Biggs, J. S. Bentley, J. C. Price, S. C. Clement, M. J. Clement, S. R. Long, and J. S. Griffiths. 2012. Rhizobial

- plasmids that cause impaired symbiotic nitrogen fixation and enhanced host invasion. *Mol. Plant Microbe Interact.* 25:1026-1033.
- Darriba, D., G. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9:772.
- Denton, M. D., C. R. Hill, W. D. Bellotti, and D. R. Coventry. 2007. Nodulation of *Medicago truncatula* and *Medicago polymorpha* in two pastures of contrasting soil pH and rhizobial populations. *Applied Soil Ecology* 35:441-448.
- Dickie, I. A., J. L. Bufford, R. C. Cobb, M. L. Desprez-Loustau, G. Grelet, P. E. Hulme, J. Klironomos, A. Makiola, M. A. Nunez, A. Pringle, et al. 2017. The emerging science of linked plant-fungal invasions. *New Phytol.* 215:1314-1332.
- Dlugosch, K. M., and I. M. Parker. 2008. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol. Ecol.* 17:431-449.
- Dudenhöffer, J.-H., A. Ebeling, and A.-M. Klein. 2018. Beyond biomass: soil feedbacks are transient over plant life stages and alter fitness. *Journal of Ecology* 106:230-241.
- Feis, M. E., M. A. Goedknecht, D. W. Thielges, C. Buschbaum, and K. M. Wegner. 2016. Biological invasions and host-parasite coevolution: different coevolutionary trajectories along separate parasite invasion fronts. *Zoology* 119:366-374.
- Ferlian, O., N. Eisenhauer, M. Aguirrebengoa, M. Camara, I. Ramirez-Rojas, F. Santos, K. Tanalgo, and M. P. Thakur. 2018. Invasive earthworms erode soil biodiversity: a meta-analysis. *Journal of Animal Ecology* 87:162-172.
- Friesen, M. L. 2012. Widespread fitness alignment in the legume-rhizobium symbiosis. *New Phytol.* 194:1096-1111.
- Garau, G., W. G. Reeve, L. Brau, P. Deiana, R. J. Yates, D. James, R. Tiwari, G. W. O'Hara, and J. G. Howieson. 2005. The symbiotic requirements of different *Medicago* spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. *Plant Soil* 276:263-277.
- Getman-Pickering, Z. L., C. P. terHorst, S. M. Magnoli, and J. A. Lau. 2018. Evolution of increased *Medicago polymorpha* size during invasion does not result in increased competitive ability. *Oecologia* 188:203-212.
- Gomulkiewicz, R., D. M. Drown, M. F. Dybdahl, W. Godsoe, S. L. Nuismer, K. M. Pepin, B. J. Ridenhour, C. I. Smith, and J. B. Yoder. 2007. Dos and don'ts of testing the geographic mosaic theory of coevolution. *Heredity* 98:249-258.
- Guy-Haim, T., D. A. Lyons, J. Kotta, H. Ojaveer, A. M. Queiros, E. Chatzinikolaou, C. Arvanitidis, S. Como, P. Magni, A. J. Blight, et al. 2018. Diverse effects of invasive ecosystem engineers on marine biodiversity and ecosystem functions: a global review and meta-analysis. *Global Change Biol.* 24:906-924.
- Harrison, T. L., C. W. Wood, I. L. Borges, and J. R. Stinchcombe. 2017a. No evidence for adaptation to local rhizobial mutualists in the legume *Medicago lupulina*. *Ecology and Evolution* 7:4367-4376.
- Harrison, T. L., C. W. Wood, K. D. Heath, and J. R. Stinchcombe. 2017b. Geographically structured genetic variation in the *Medicago lupulina*-*Ensifer* mutualism. *Evolution* 71:1787-1801.
- Hartig, F. 2019. DHARMA: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.1.
- Hassani, M. A., P. Durán, and S. Hacquard. 2018. Microbial interactions within the plant holobiont. *Microbiome* 6:58.
- Heath, K. D. 2010. Intergenomic epistasis and coevolutionary constraint in plants and rhizobia. *Evolution* 64:1446-1458.
- Heath, K. D., P. V. Burke, and J. R. Stinchcombe. 2012. Coevolutionary genetic variation in the legume-rhizobium transcriptome. *Mol. Ecol.* 21:4735-4747.
- Heath, K. D., and S. L. Nuismer. 2014. Connecting functional and statistical definitions of genotype by genotype interactions in coevolutionary studies. *Frontiers in Genetics* 5:77.
- Helliwell, E. E., J. Faber-Hammond, Z. C. Lopez, A. Garoutte, E. von Wettberg, M. L. Friesen, and S. S. Porter. 2018. Rapid establishment of a flowering cline in *Medicago polymorpha* after invasion of North America. *Mol. Ecol.* 27:4758-4774.
- Henriques, G. J. B., and M. M. Osmond. 2020. Cooperation can promote rescue or lead to evolutionary suicide during environmental change. *Evolution* 74:1255-1273.
- Irimia, R. E., S. M. M. Lopes, G. Sotes, L. A. Cavieres, Ö. Eren, C. J. Lortie, K. French, J. L. Hierro, C. Rosche, R. M. Callaway, et al. 2019. Biogeographic differences in the allelopathy of leaf surface extracts of an invasive weed. *Biological Invasions* 21:3151-3168.
- Kiers, E. T., and R. F. Denison. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annu. Rev. Ecol. Evol. Syst.* 39:215-236.
- Klinger, C. R., J. A. Lau, and K. D. Heath. 2016. Ecological genomics of mutualism decline in nitrogen-fixing bacteria. *Proceedings of the Royal Society B: Biological Sciences* 283:20152563.
- Klironomos, J. N. 2003. Variation in plant response to native and exotic arbuscular mycorrhizal fungi. *Ecology* 84:2292-2301.
- Kolbe, J. J., A. Larson, J. B. Losos, and K. de Queiroz. 2008. Admixture determines genetic diversity and population differentiation in the biological invasion of a lizard species. *Biol. Lett.* 4:434-437.
- Koskella, B., and J. Bergelson. 2020. The study of host-microbiome (co)evolution across levels of selection. *Philosophical Transactions of the Royal Society B: Biological Sciences* 375:20190604.
- Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870-1874.
- Lavergne, S., and J. Molofsky. 2007. Increased genetic variation and evolutionary potential drive the success of an invasive grass. *Proceedings of the National Academy of Science USA* 104:3883-3888.
- Le Roux, J. J., C. Hui, J. H. Keet, and A. G. Ellis. 2017. Co-introduction vs ecological fitting as pathways to the establishment of effective mutualisms during biological invasions. *New Phytol.* 215:1354-1360.
- Li, W., Y. Zheng, L. Zhang, Y. Lei, Y. Li, Z. Liao, Z. Li, and Y. Feng. 2020. Postintroduction evolution contributes to the successful invasion of *Chromolaena odorata*. *Ecology and Evolution* 10:1252-1263.
- Lopez, Z. C., M. L. Friesen, E. von Wettberg, L. New, and S. Porter. 2020. Microbial mutualist distribution limits spread of the invasive legume *Medicago polymorpha*. *Biological Invasions*. <https://doi.org/10.1007/s10530-020-02404-4>
- Lu-Irving, P., J. G. Harenčár, H. Sounart, S. R. Welles, S. M. Swope, D. A. Baltrus, and K. M. Dlugosch. 2019. Native and invading yellow starthistle (*Centaurea solstitialis*) microbiomes differ in composition and diversity of bacteria. *mSphere* 4:e00088-19.
- Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland, MA.
- Moeller, H. V., I. A. Dickie, D. A. Peltzer, and T. Fukami. 2015. Mycorrhizal co-invasion and novel interactions depend on neighborhood context. *Ecology* 96:2336-2347.
- Ochocki, B. M., and T. E. Miller. 2017. Rapid evolution of dispersal ability makes biological invasions faster and more variable. *Nat. Commun.* 8:14315.
- Oldroyd, G. E., J. D. Murray, P. S. Poole, and J. A. Downie. 2011. The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45:119-144.
- Oono, R., C. G. Anderson, and R. F. Denison. 2011. Failure to fix nitrogen by non-reproductive symbiotic rhizobia triggers host sanctions that

- reduce fitness of their reproductive clonemates. *Proceedings of the Royal Society B: Biological Sciences* 278:2698-2703.
- Osnas, E. E., P. J. Hurtado, and A. P. Dobson. 2015. Evolution of pathogen virulence across space during an epidemic. *Am. Nat.* 185:332-342.
- Owen, M. A., and D. C. Lahti. 2020. Rapid evolution by sexual selection in a wild, invasive mammal. *Evolution* 74:740-748.
- Pan, H., and D. Wang. 2017. Nodule cysteine-rich peptides maintain a working balance during nitrogen-fixing symbiosis. *Nature Plants* 3: 17048.
- Pankoke, H., L. J. Tewes, S. Matties, I. Hensen, M. Schädler, S. Ebeling, H. Auge, and C. Müller. 2019. Pre-adaptations and shifted chemical defences provide *Buddleja davidii* populations with high resistance against antagonists in the invasive range. *Biological Invasions* 21:333-347.
- Porter, S. S., J. J. Faber-Hammond, and M. L. Friesen. 2018. Coinvading symbiotic mutualists of *Medicago polymorpha* retain high ancestral diversity and contain diverse accessory genomes. *FEMS Microbiol. Ecol.* 94:fix168.
- Porter, S. S., and K. J. Rice. 2013. Trade-offs, spatial heterogeneity, and the maintenance of microbial diversity. *Evolution* 67:599-608.
- Porter, S. S., and E. L. Simms. 2014. Selection for cheating across disparate environments in the legume-rhizobium mutualism. *Ecology Letters* 17:1121-1129.
- Porter, S. S., M. L. Stanton, and K. J. Rice. 2011. Mutualism and adaptive divergence: co-invasion of a heterogeneous grassland by an exotic legume-rhizobium symbiosis. *PLoS One* 6:e27935.
- Price, P. A., H. R. Tanner, B. A. Dillon, M. Shabab, G. C. Walker, and J. S. Griffiths. 2015. Rhizobial peptidase HrrP cleaves host-encoded signaling peptides and mediates symbiotic compatibility. *Proc. Natl. Acad. Sci.* 112:15244-15249.
- Qiao, H., W. Liu, Y. Zhang, Y. Y. Zhang, and Q. Q. Li. 2019. Genetic admixture accelerates invasion via provisioning rapid adaptive evolution. *Mol. Ecol.* 28:4012-4027.
- Queller, D. C. 2014. Joint phenotypes, evolutionary conflict and the fundamental theorem of natural selection. *Philosophical Transactions of the Royal Society B: Biological Sciences* 369:20130423.
- R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Richardson, D. M., N. Allsopp, C. M. D'Antonio, S. J. Milton, and M. Rejmanek. 2000. Plant invasions—the role of mutualisms. *Biol. Rev. Camb. Philos. Soc.* 75:65-93.
- Richardson, D. M., and M. Rejmanek. 2011. Trees and shrubs as invasive alien species: a global review. *Diversity and Distributions* 17:788-809.
- Rodríguez-Echeverría, S., J. A. Crisóstomo, C. Nabais, and H. Freitas. 2008. Belowground mutualists and the invasive ability of *Acacia longifolia* in coastal dunes of Portugal. *Biological Invasions* 11:651-661.
- Rodríguez-Echeverría, S., S. Fajardo, B. Ruiz-Diez, and M. Fernandez-Pascual. 2012. Differential effectiveness of novel and old legume-rhizobia mutualisms: implications for invasion by exotic legumes. *Oecologia* 170:253-261.
- Seifert, E. K., J. D. Bever, and J. L. Maron. 2009. Evidence for the evolution of reduced mycorrhizal dependence during plant invasion. *Ecology* 90:1055-1062.
- Shelby, N., R. P. Duncan, W. H. Van Der Putten, K. J. McGinn, C. Weser, and P. E. Hulme. 2016. Plant mutualisms with rhizosphere microbiota in introduced versus native ranges. *Journal of Ecology* 104:1259-1270.
- Simonsen, A. K., R. Dinnage, L. G. Barrett, S. M. Prober, and P. H. Thrall. 2017. Symbiosis limits establishment of legumes outside their native range at a global scale. *Nat. Commun.* 8:14790.
- Simonsen, A. K., and J. R. Stinchcombe. 2014. Standing genetic variation in host preference for mutualist microbial symbionts. *Proceedings of the Royal Society B: Biological Sciences* 281:20142036.
- Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312-1313.
- terHorst, C. P., C. Wirth, and J. A. Lau. 2018. Genetic variation in mutualistic and antagonistic interactions in an invasive legume. *Oecologia* 188:159-171.
- Timmers, A. C., E. Soupène, M.-C. Auriac, F. de Billy, J. Vasse, P. Boistard, and G. Truchet. 2000. Saprophytic intracellular rhizobia in alfalfa nodules. *Mol. Plant Microbe Interact.* 13:1204-1213.
- Vanbergen, A. J., A. Espíndola, and M. A. Aizen. 2018. Risks to pollinators and pollination from invasive alien species. *Nat. Ecol. Evol.* 2:16-25.
- van Kleunen, M., O. Bossdorf, and W. Dawson. 2018. The ecology and evolution of alien plants. *Annu. Rev. Ecol. Evol. Syst.* 49:25-47.
- Vincent, J. M. 1970. A manual for the practical study of the root-nodule bacteria. Blackwell Scientific, Oxford, U.K.
- Wandrag, E. M., C. Birnbaum, M. M. Klock, L. G. Barrett, and P. H. Thrall. 2020. Availability of soil mutualists may not limit non-native *Acacia* invasion but could increase their impact on native soil communities. *J. Appl. Ecol.* 57:786-793.
- Weese, D. J., K. D. Heath, B. T. M. Dentinger, and J. A. Lau. 2015. Long-term nitrogen addition causes the evolution of less-cooperative mutualists. *Evolution* 69:631-642.
- West, S. A., E. T. Kiers, E. L. Simms, and R. F. Denison. 2002. Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proceedings of the Royal Society of London B* 269:685-694.
- Wing, J. E. 1912. Alfalfa farming in America. Sanders Publishing Company, Chicago, IL.
- Yoshida, T., K. Goka, F. Ishihama, M. Ishihara, and S.-i. Kudo. 2007. Biological invasion as a natural experiment of the evolutionary processes: introduction of the special feature. *Ecol. Res.* 22:849-854.
- Zenni, F. D., I. A. Dickie, M. J. Wingfield, H. Hirsch, C. J. Crous, L. A. Meyerson, T. I. Burgess, T. G. Zimmermann, M. M. Klock, E. Siemann, et al. 2017. Evolutionary dynamics of tree invasions: complementing the unified framework for biological invasions. *AoB Plants* 9:plw085.

Associate Editor: D. Drown

Handling Editor: D. Hall

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Genotypes of *Ensifer medicae* and *Medicago polymorpha* used in the greenhouse experiment.

Table S2. Sampling information for the 36 field sites (populations) where *Medicago polymorpha* and *Ensifer medicae* were sampled for this study, including the number of *E. medicae* strains identified as *hrrP*+ or *hrrP*−.

Figure S1. Mutualism traits estimating mutualism benefits to *Medicago polymorpha* (left panels) and *Ensifer medicae* (right panels).

Figure S2. *Medicago polymorpha* leaf count is higher in the invaded range than the native range.

Figure S3. *Medicago polymorpha* plant size (A, leaf count; B, shoot mass) increases with the frequency of *hrrP*+ *Ensifer medicae* in their home population.