1	Novel plant-microbe interactions: rapid evolution of a legume-rhizobium mutualism in restored
2	prairies
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# 17 Abstract

18	1.	When plants colonize new habitats, the novel interactions they form with new
19		mutualists or enemies can immediately affect plant performance. These novel
20		interactions also may provoke rapid evolutionary responses and can be ideal scenarios
21		for investigating how species interactions influence plant evolution.
22	2.	To explore how mutualists influence the evolution of colonizing plant populations, we
23		capitalized on an experiment in which two former agricultural fields were seeded with
24		identical prairie seed mixes in 2010. Six years later, we compared how populations of
25		the legume Chamaecrista fasciculata from these sites and their original (shared) source
26		population responded to nitrogen-fixing rhizobia from the restoration sites in a
27		greenhouse reciprocal cross-inoculation experiment.
28	3.	We found that the two populations differed both from their original source population
29		and from each other in the benefits they derive from rhizobia, and that one population
30		has evolved reduced allocation to rhizobia (i.e., forms fewer rhizobium-housing
31		nodules).
32	4.	Synthesis. Our results suggest that these plant populations have evolved different ways
33		of interacting with rhizobia, potentially in response to differences in rhizobium quality
34		between sites. Our study illustrates how microbial mutualists may shape plant evolution
35		in new environments and highlights how variation in microbial mutualists potentially
36		may select for different evolutionary strategies in plant hosts.
37		

38 Keywords

Chamaecrista fasciculata, coevolution, contemporary evolution, ecological restoration, legume rhizobium mutualism, rapid evolution, species interactions

41

# 42 Introduction

43 Plant populations colonizing new habitats inevitably form novel interactions with 44 mutualists, enemies, and competitors (Richardson et al. 2000, Levine et al. 2004, Traveset and 45 Richardson 2014). While much work has focused on the ecological effects of these interactions 46 (Mitchell et al. 2006), mutualists, enemies, and competitors also may act as strong agents of 47 natural selection on colonizing populations (e.g. Lambrinos 2004). Microbial mutualists may be particularly important to the success of colonizing plants (Parker 2001, Parker et al. 2006, 48 49 Rodríguez-Echeverría et al. 2009, Rodríguez-Echeverría et al. 2012, Porter et al. 2011, Dawson 50 and Schrama 2016, Lau and Suwa 2016), and have the potential to act as strong agents of 51 selection given their ability to influence plant fitness (Parker 1995, Rúa et al. 2016). These 52 mutualists can benefit plants by increasing access to nutrients (Kiers and Denison 2008), mediating abiotic stress (Rodriguez et al. 2008, Dimkpa et al. 2009, Yang et al. 2017), and 53 54 protecting plants from herbivory (Clay 1996). However, mutualist partners vary in quality, 55 displaying both intra- and interspecific variation in the benefits they provide to their partners 56 (Burdon et al. 1999, Heath 2010, Hoeksema 2010, Heath and Stinchcombe 2014, Weese et al. 57 2015), and the outcomes of plant-mutualist interactions can (e.g. Heath and Tiffin 2007, Barret et 58 al. 2016, Rúa et al. 2016), but do not always (e.g. Harrison et al. 2017) depend on the genotypes 59 of both interacting partners. As a result, colonizing plant populations are likely to encounter 60 microbial mutualists that differ in quality or compatibility from mutualists at their native sites 61 (Shelby et al. 2016).

62 Many plant species also show intraspecific variation in the benefits they derive from their 63 microbial mutualists. For example, several studies of legume-rhizobium interactions have shown 64 that some plant genotypes or populations benefit more from rhizobia than others (Parker 1995, Heath and Tiffin 2007, Heath 2010, Keller and Lau 2018). Variation among populations in plant 65 66 dependence on microbes (i.e., the magnitude of benefit from association) can evolve in response 67 to variation in the presence or abundance of mutualists or from abiotic factors such as resource 68 availability that influence the outcome of the interaction. For example, an invasive plant appears 69 to have evolved reduced dependence on mycorrhizae due to lack of compatible mutualists in the 70 introduced range (Seifert et al. 2009). Similarly, an Andropogon gerardii population growing in a high nutrient site where resource mutualists may be less necessary evolved reduced 71 72 dependence on arbuscular mycorrhizal fungi compared to a population in a low nutrient site 73 (Schultz et al. 2001).

74 Here, we investigated plant responses to novel microbial interactions in recently restored 75 prairies. Prairie restorations provide an ideal opportunity to examine novel plant-microbe interactions because plant species are typically planted into highly disturbed sites inhabited by 76 77 populations of microbial mutualists with which they are unlikely to share a recent evolutionary 78 history. We examined populations of the annual legume Chamaecrista fasciculata that originated 79 from the same source population, along with their associated nitrogen-fixing mutualist rhizobia, 80 in two restored prairies that differ in plant community composition and edaphic properties to 81 determine whether plants have evolved novel interactions with local rhizobia in the six years 82 since they were established, and whether this evolution may be due to differences in rhizobium 83 quality between the two restoration sites. In a greenhouse reciprocal cross-inoculation

experiment, we addressed the following questions: Have restored plant populations evolved
differences in 1) the benefits they derive from rhizobia and 2) their allocation to rhizobia?

87 Materials and Methods

#### 88 *Study system*

89 Chamaecrista fasciculata Michx. is an annual legume native to eastern North America 90 commonly found in prairies and disturbed sites. *Chamaecrista* forms facultative mutualistic 91 interactions with rhizobia, such as *Bradyrhizobium* spp., which provide plants fixed nitrogen in 92 exchange for carbon. For this study, we used *Chamaecrista* populations from two recently 93 restored prairies in southwest Michigan, Lux Arbor (42°28'23" N, 85°26'50" W) and Marshall (42°26'37" N, 85°18'34" W). These two former agricultural fields were planted with identical 94 95 prairie seed mixes (containing 19 grass and forb species) in 2010 using a no-till seed drill. The 96 Chamaecrista seeds in this mix were cultivated by Shooting Star Native Seeds, a commercial 97 seed supplier in Houston County, Minnesota. A portion of the seed mix was saved (hereafter 98 referred to as the 'original source'), stored in a plastic mesh bag in a temperature-controlled 99 storage room (20-23°C). The microbial community was not manipulated in either site. Despite 100 the similar treatment of each site and the identical seed mixes used to plant them, they have 101 differed in community composition every year since they were planted, and *Chamaecrista* 102 biomass is consistently higher (2-12 times greater, depending on year) at the Lux site than at 103 Marshall. The sites also differ in underlying abiotic factors, such as soil phosphorus (an average 104 of 54.54ppm and 23.78ppm at Marshall and Lux, respectively), and available soil nitrogen, with 105 the Marshall site having twice as much available soil ammonium and nitrate as Lux (2.3g N/kg 106 soil vs. 1.3g N/kg soil respectively; Stahlheber et al. 2016), although, both sites are fairly low in 107 N (Kellogg Biological Station LTER pers. comm.). A previous study of *Chamaecrista* 

populations at these sites conducted in 2016 found genetic differentiation between *Chamaecrista* populations in root nodule (structures that house rhizobia) production, with Lux plants producing significantly more root nodules than Marshall plants (Magnoli 2018), suggesting that these plant populations interact differently with rhizobia.

112 In 2015 we collected seeds from 100 haphazardly selected *Chamaecrista* individuals at 113 each prairie site. This was a maximum of six *Chamaecrista* generations after the restorations 114 were planted—*Chamaecrista* has been shown to have a limited seed bank, with >90% of viable 115 seeds in a seed bank study germinating in the first year (Fenster 1991). We grew these field-116 collected seeds, along with seeds from the original source, for one generation in the greenhouse 117 in 2017 to minimize maternal effects. We had low germination success of the original source 118 seed (7% germination compared to about 95% germination of the two field collected 119 populations). While this is not unexpected given the age of the seeds, if mortality was not 120 random with respect to the traits considered in this study, then selective mortality during seed storage may influence comparisons of the original source population to the two restored 121 122 populations. Because of this concern we focus primarily on comparisons between the two 123 restored populations in the discussion. We hand-pollinated plants, using one plant in each 124 population as a pollen donor to all the other plants in its population on a given day, so that each 125 plant was eventually crossed with every other plant in its population. We pollinated this way to 126 ensure outcrossing and maximize genetic diversity in our sample populations. We used the 127 offspring from these greenhouse-reared plants in the experiment described below. 128 To isolate rhizobium strains, in summer 2017 we collected soil cores (2cm core to 10cm

depth) at 10m intervals along a 200m transect through the middle of each site, and homogenized

130 the samples from each site. We inoculated 10 Lux seedlings, 10 Marshall seedlings, and 10 131 original source seedlings grown in potting soil (Sunshine Mix #5; Sun Gro Horticulture Canada 132 Ltd., Alberta, Canada) in 656 mL Deepots<sup>™</sup> (Stuewe & Sons Inc., Corvallis, OR, USA) in the 133 greenhouse with 2g of either Lux or Marshall soil (60 total seedlings). We did not sterilize 134 potting soil prior to use, as *Chamaecrista* grown in this soil in previous studies has rarely 135 nodulated, indicating no contamination with Chamaecrista-compatible rhizobium strains. We 136 placed plants approximately 15cm apart in racks and avoided cross-contamination by watering 137 carefully to prevent splashing between pots. Both previous and simultaneous studies on this 138 species with similar spacing had minimal (1-3%) contamination of uninoculated controls. Four 139 weeks after inoculation, we harvested two randomly selected root nodules from each plant, and 140 attempted to isolate rhizobia strains from each. We surface-sterilized individual nodules by 141 dipping them in 100% ethanol followed by 1 minute in commercial bleach, followed by a water rinse, and then crushed them and plated them onto tryptone yeast (TY) agar plates (Somasegaran 142 143 and Hoben 1994). We re-streaked strains onto additional TY plates until we obtained single colonies, eventually isolating 21 single strains of Lux rhizobia and 27 of Marshall rhizobia. We 144 145 isolated strains from nodules produced by individuals from all three plant populations to avoid 146 any biases in rhizobium selection by the different plant populations. Subsequent analyses showed 147 that which plant population rhizobium strains were isolated from had no effect on rhizobia or 148 plant performance (data not shown).

149 Reciprocal cross-inoculation experiment

To investigate differences between plant populations in benefits from rhizobia, and variation among sites in rhizobium quality, we conducted a fully-factorial greenhouse experiment manipulating plant population and rhizobium presence and source. We surface153 sterilized *Chamaecrista* seeds from each of the three populations (Lux, Marshall, original) in 154 75% ethanol for one minute and germinated them in Petri dishes with distilled water. After 155 germination, we transferred individual seedlings to 656 mL Deepots<sup>TM</sup> filled with the same brand 156 of unsterilized potting soil described above. One week later we inoculated seedlings with 1ml of a mixture of Lux or Marshall rhizobium strains in liquid culture (c.  $2.5 \times 10^6$  cells based on 157 OD670), or sterile liquid culture. Rhizobium cultures were grown in Modified Arabinose 158 159 Gluconate (MAG) liquid culture (van Berkum 1990) at 30°C for 48 hours. Each rhizobium 160 mixture was comprised of nine strains, three isolated from nodules produced by Lux seedlings, 161 three from Marshall seedlings, and three from original source seedlings, as this was the greatest 162 number of strains we could use to have an equal number of strains isolated from seedlings from 163 each population (these were selected at random from the single strains we successfully isolated). 164 Cell density was measured with a spectrophotometer and standardized by diluting individual 165 strain cultures with sterile media before combining. Because sites differed in soil nitrogen 166 availability and the outcomes of legume-rhizobium mutualisms can be sensitive to N availability 167 (Thrall et al. 2007, Kiers et al. 2010, Regus et al. 2017), we conducted these experiments in two 168 soil nitrogen levels. Plants were fertilized with ammonium nitrate at either lower (1.3g/kg soil) or 169 higher (2.3g/kg soil) levels to approximate total available soil nitrogen at the Lux and Marshall 170 sites, respectively. We fertilized in three intervals, starting three weeks after planting and every 171 following two weeks. Each plant population/rhizobium/nitrogen treatment was replicated 30 172 times [3 plant populations (Lux, Marshall, original) x 3 rhizobia (Lux, Marshall, none) x 2 N 173 (lower, higher) x 30 replicates = 540 total plants].

While inoculating plants with a mixture of rhizobia from each site simulates a diverse
rhizobium community like those typically experienced by legumes in nature, it masks any

176 differences between individual rhizobium strains. To determine if specific strains were driving 177 site-specific effects of rhizobia on plants, we also inoculated plants from the two extant sites 178 (Lux and Marshall) with each single strain used in the rhizobium mixtures. We did not include a 179 nitrogen treatment, but fertilized plants at the low nitrogen level described above (2 plant 180 populations x 18 rhizobium strains x 5 replicates = 180 total plants). Results from single strain 181 inoculations were qualitatively similar to those from the multi-strain inoculations (although small 182 samples sizes limited statistical power), so we only present results from the multi-strain analyses 183 in the main text (see Supplementary Material for detailed methods and Fig. S1 for single-strain 184 results).

185 We harvested above and belowground biomass nine weeks after initial planting, just as 186 plants were beginning to flower. We counted root nodules and haphazardly selected 10 nodules 187 from each plant and weighed them to calculate mean nodule mass. One plant from the no rhizobia control treatment formed a single nodule (suggesting very little contamination between 188 189 rhizobia treatments) and this plant was excluded from analyses. We dried above and 190 belowground biomass at 60° C for 48 hours and weighed it to use as an estimate of plant fitness, 191 as biomass has been shown to be positively correlated with seed production in this species 192 (Galloway and Fenster 2001). We did not measure seed set directly because although 193 *Chamaecrista* is self-compatible, seed production is substantially reduced in the absence of 194 pollinators. Statistical analyses 195

All analyses were conducted in R v.3.3.2 (R Core Team 2018) using mixed models in the
 *lme4* package (Bates et al. 2015). For all models, we used diagnostic plots to visually check

model assumptions (normality of residuals, homogeneity of variances). We conducted thefollowing analyses to address our specific questions:

200 1) Do plant populations differ in the benefits they derive from rhizobia? We compared 201 total biomass of inoculated and uninoculated plants from all populations using a model with total 202 plant biomass (above + belowground biomass) as the response variable, plant population, 203 rhizobia (Lux, Marshall, or no rhizobia), nitrogen, and interactions between these factors as fixed 204 effects, and block (greenhouse position) as a random effect. We tested significance of fixed 205 effects using type III sums of squares in the Anova function in the *car* package (Fox and 206 Weisberg 2011) with sum contrasts and estimated marginal means using the *emmeans* package 207 (Lenth 2018). Upon finding a significant plant population x rhizobia x nitrogen effect on 208 biomass, we conducted two separate post-hoc comparison tests. To determine whether plant 209 populations differed from each other in biomass production when inoculated with a given 210 rhizobium population in a given nitrogen environment, we used Tukey's HSD post-hoc multiple 211 comparisons tests. To determine whether there were significant differences in biomass 212 production between inoculated and uninoculated plants from a given population in a given 213 nitrogen environment (a way to examine whether a population benefits from rhizobia), we used 214 pairwise post-hoc contrasts comparing a population's mean biomass when inoculated with 215 rhizobia to its mean biomass when un-inoculated using the dunnettx method for p-value 216 adjustment.

2) Do plant populations differ in their allocation to rhizobia? To determine whether Lux
and Marshall plant populations differ in their allocation to rhizobia and whether rhizobium
populations differ in traits relevant to their mutualism with *Chamaecrista*, we compared the
number of nodules and estimated mean nodule biomass plants produced when inoculated with

different rhizobium populations. To analyze nodule number data, we used a generalized linear mixed model with a Poisson distribution and plant population, rhizobia, and nitrogen and all interactions between these variables as fixed effects, and block as a random effect. For estimated mean nodule mass we used a linear mixed model with the same predictor variables, and logtransformed nodule biomass to better conform to model assumptions. For both models, we tested significance of fixed effects using Anova as described above and used Tukey's HSD post-hoc multiple comparisons tests to examine differences between groups.

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### 229 Results

230 Do plant populations differ in the benefits they derive from rhizobia? Plant biomass 231 varied between populations, and depended on rhizobia and nitrogen (plant population x rhizobia 232 x nitrogen F<sub>4,483</sub>=3.63, p=0.006; Fig. 1, Table S1). Comparisons of biomass production between 233 plant populations showed that when plants were un-inoculated, the original source population 234 produced significantly more biomass than the other two populations, but only under lower 235 nitrogen conditions (Fig. 1a). Additionally, the Marshall plant population produced significantly 236 more biomass than the Lux population when inoculated with rhizobia from the Marshall site 237 under both nitrogen conditions and tended to produce more biomass than the original population 238 (p=0.07) but only under lower nitrogen conditions. There were no differences in biomass 239 between plant populations inoculated with rhizobia from the Lux site. 240 Comparisons of the biomass of inoculated to un-inoculated plants in a given population 241 showed that under lower nitrogen conditions, rhizobia from the Marshall site benefited the

243 with rhizobia from the Marshall site had significantly higher biomass than un-inoculated

Marshall plant population but not the other plant populations (that is, Marshall plants inoculated

Marshall plants; Fig.2a). Rhizobia from the Lux site had a negative effect on the original source population under lower nitrogen conditions (original plants had significantly higher biomass when un-inoculated than when inoculated with rhizobia from the Lux site), but had no effect on the other populations. Under higher nitrogen conditions there were no significant differences between inoculated and un-inoculated plants within a given population, but rhizobia from the Marshall site tended to have a positive effect on Marshall plants and a negative effect on Lux plants (Fig. 2b).

251 Do plant populations differ in their allocation to rhizobia? Plant populations differed in 252 their allocation to rhizobia (nodule number), although the magnitude of difference depended on the rhizobium population (plant population x rhizobia:  $\chi^2$ =9.44, p=0.009; Fig. 3a; Table S2). The 253 Lux plant population produced significantly fewer nodules than the other two populations when 254 255 inoculated with rhizobia from the Lux site and fewer nodules than Marshall plants when 256 inoculated with rhizobia from the Marshall population. Plants grown under lower nitrogen 257 conditions produced more nodules than those grown under higher nitrogen conditions regardless 258 of rhizobia origin, but the magnitude of the nitrogen effect on nodule number differed between 259 populations, with the Lux population again tending to produce fewer nodules than the other 260 populations, but especially under higher nitrogen conditions (plant population x nitrogen 261  $\chi^2$ =8.78, p=0.01; Fig. 3b).

Similar to the nodule number results, plants inoculated with rhizobia from the Lux site produced bigger nodules than those inoculated with rhizobia from the Marshall site (rhizobia origin  $F_{1,291}=112.46$ , p<0.0001; Fig. 3c, Table S3), but unlike nodule number, this effect did not differ between populations or between nitrogen conditions (Fig. 3d). 266 Our single strain inoculations indicated that several rhizobium strains (mostly from the 267 Marshall site) were ineffective (that is, plants inoculated with these strains did not produce 268 nodules; see Supplement results section for more details).

269

## 270 Discussion

271 *Chamaecrista* populations from two recently restored prairies, only 15km apart, which 272 were planted using the same source population six years prior to this experiment, have rapidly 273 evolved altered interactions with rhizobia. The two plant populations differ both from their 274 original source population and from each other in the benefits they derive from rhizobia and the 275 number of root nodules they produce, although the magnitude of these differences depends on 276 nitrogen conditions and rhizobia population. Our findings suggest: 1) seeds used in restorations 277 may not be optimally adapted to rhizobia in the restored sites as original source seeds derived no 278 benefit from rhizobia and 2) restored plant populations can, but do not always, rapidly evolve to 279 benefit from local mutualists.

280 We have two main hypotheses to explain why one plant population (Marshall) evolved to 281 benefit more from its local rhizobia population while the other (Lux) did not. First, differences in 282 available soil nitrogen between the two sites could directly influence how plants evolve to interact with rhizobia. Second, differences between the rhizobia communities themselves could 283 284 influence the evolution of plant-rhizobia interactions, especially if the rhizobia communities 285 differ in quality (i.e. the benefits they provide to plants). Rhizobia communities may differ 286 between sites as a result of differences in site use history prior to restoration, differences in post-287 restoration plant community composition, or soil nitrogen availability. We discuss each of these 288 hypotheses in more detail below.

289 The Lux restoration site has approximately half the available soil ammonium and nitrate 290 of the Marshall site, and as a result, we expected that the Lux plant population, evolving in a site 291 with lower soil nitrogen, would evolve to benefit more from rhizobia than the Marshall 292 population, as lower soil resource availability is predicted to increase plant dependence on 293 microbial mutualists (e.g., Schultz et al. 2001). We found the opposite pattern, however, with the 294 Lux population benefiting less from rhizobia from its homesite than the Marshall population. 295 This result suggests that the differences in available nitrogen between the two sites may not be 296 great enough to have driven the differences in rhizobia benefit we detect between plant 297 populations. While there is a two-fold difference in soil nitrogen between the sites (2.3g N/kg 298 soil vs. 1.3g N/kg soil), both sites are considered to be fairly low N. In our experiment there were 299 no differences in average plant biomass between nitrogen treatments (although nitrogen did 300 affect nodule numbers in ways that conform to findings in other studies (e.g. Weese et al. 2015; 301 Cauwenberghe et al. 2016; Regus et al. 2017), suggesting that the differences in soil nitrogen 302 between the two sites may not be big enough to greatly affect plant growth and/or that nitrogen 303 was never limiting even in the low N treatments.

304 It may be more likely that the rhizobia communities in these sites, which appear to differ 305 in the benefits they provide to plants, influenced the evolution of plant-rhizobium interactions. 306 Our findings (Fig. 1) suggest that rhizobia from the Marshall site tend to provide more benefits 307 to plants than Lux rhizobia, at least for their local plant population. The Marshall plant 308 population may have adapted to the more beneficial rhizobia at the Marshall site, while the Lux 309 population did not evolve to capitalize on the Lux rhizobia that appear to provide no fitness 310 benefit. The Lux plant population even evolved to reduce allocation to rhizobium symbionts. 311 Similarly, in a study of invasive populations of *Hypericum perforatum*, Seifert et al. (2009)

312 found evolution of decreased dependence on mycorrhizae, which may have been driven by a lack 313 of suitable microbial mutualists in the invaded range, suggesting that variation in microbial 314 quality or presence can influence the evolution of newly established plant populations. 315 Several factors could lead to the differences we found in the rhizobia communities at our 316 two restoration sites, but pre-restoration site history may be the most likely to have influenced 317 these differences. Although both sites were planted as prairies in 2010, their management 318 differed prior to restoration, which likely affected soil properties and plant community 319 composition, which in turn would influence the microbial community (Koziol et al. 2018). Both 320 sites have a history of row crop agriculture prior to restoration (likely corn/soy rotations), 321 meaning rhizobia compatible with *Chamaecrista* were likely present (soy and *Chamaecrista* both 322 associate with *Bradyrhizobium*, although we do not have records of whether soy crops were 323 actively inoculated). However, the Marshall site was used for agriculture up to 1987, when it was 324 planted with perennial grass species and put in the USDA conservation reserve program 325 (Stahlheber et al. 2016). In contrast, the Lux site had been used for row crops (including corn, soy, wheat, and alfalfa) continuously for at least 70 years prior to restoration, meaning it was 326 327 growing with soybean hosts and receiving fertilizer inputs for over twenty years (1987-2009) 328 when the Marshall site was not. Growing in the presence of soy hosts could have selected for 329 strains that are less beneficial for alternate hosts like Chamaecrista, as different legume species 330 have been shown to be compatible with the same rhizobia but vary in the benefits they derive 331 from them (Pahua et al. 2018). And while the Lux site now has lower soil nitrogen availability, 332 prior fertilizer inputs could have led to the evolution of less beneficial mutualists that might still persist today (Johnson 1993, Corkidi et al. 2002, Weese et al. 2015, but see Schmidt et al. 2017). 333

334 Post-restoration differences between the two sites could also influence rhizobia 335 communities, although these may be less influential than pre-restoration site history. The current 336 nitrogen environment of these sites has the potential to influence rhizobium communities, as 337 theory predicts that higher nitrogen conditions will lead to the evolution of less beneficial 338 rhizobia (West et al. 2002, Denison and Kiers 2004, Akçay and Simms 2011). We find the 339 opposite, however, with seemingly higher-quality rhizobia at the higher-nitrogen Marshall site. 340 This suggests that the post-restoration differences in available soil nitrogen between sites may 341 not be great enough to lead to the differences we find in rhizobium communities. Differences in 342 post-restoration plant community composition between the two sites also potentially could 343 influence rhizobia communities, but this may not be likely in our system because although the 344 plant communities do differ, *Chamaecrista* is the only species known to associate with 345 Bradyrhizobium in either of these sites (although Bradyrhizobium may interact with other non-346 legumes as free-living nitrogen fixers in the rhizosphere [Antoun et al. 1998]). 347 Although we find clear evidence that the restored plant populations in our study have 348 evolved altered interactions with rhizobia, we need to be cautious about the generalizations we 349 make about rhizobium quality in this system, given the small number of strains from each site we 350 used (because we focused on plant evolutionary responses we opted for greater replication and 351 sampling of plant populations). Further work including more rhizobium strains may be necessary 352 to adequately characterize genetic differences among the two rhizobium populations, which 353 would allow us to better determine whether, as we have hypothesized, rhizobium quality 354 influenced plant evolution in this system. Additional data regarding rhizobium abundance or 355 density at each site would also help better determine differences between these communities.

356 Surprisingly, the original source population did not benefit from either rhizobium 357 population. Whether this is unique to the particular source population considered or is indicative 358 of a more general pattern of cultivated varieties evolving reduced dependence on microbial 359 mutualists due to unintentional selection during cultivation as has been observed in some 360 agronomic crops (Pérez-Jaramillo et al. 2016), remains to be seen. Additionally, the nodule 361 number results in this study contradict a field study conducted with these same plant populations 362 in 2016 (Magnoli 2018). In that study, Lux plants grown in the field produced more root nodules 363 than the Marshall population (but unlike our current study, that study had no data on the benefits 364 plant derived from rhizobia in the field). Because legume-rhizobium interactions are notoriously 365 context dependent (e.g. Lau et al. 2012), the difference between our field and greenhouse results 366 may be due to variation in environmental conditions across studies. For example, intraspecific 367 plant density has been shown to affect the costs and benefits of associating with microbial 368 mutualists, with the cost of associating with mycorrhizae increasing as plant density increases 369 (Allsopp and Stock 1992). *Chamaecrista* density in the field varies greatly, with a large 370 population at the Lux site (*Chamaecrista* can comprise 50% cover in some areas) and a relatively 371 small population at Marshall, which could explain the discrepancy between the field study and 372 this study where plants are grown in the absence of competitors.

373 Conclusion

By examining two recently restored plant populations, their original source population, and the rhizobia they associate with, we illustrate that populations can evolve divergent strategies of interaction with potential mutualists as they establish in new habitats. Interestingly, the original source population did not benefit from rhizobia and only one of the two restored plant populations evolved the ability to benefit from its local rhizobia, suggesting it may be common 379 for newly established populations to not benefit from local microbial mutualists. What limits the

380 coevolutionary dynamics of such systems requires increased attention because as anthropogenic

381 forces alter the environment and lead to more colonization events such as range expansions,

invasions, and the need to restore degraded landscapes, mutualisms between plants and microbes

383 will be disrupted and reformed in potentially novel ways.

384

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394

### **395 Author contributions**

S.M.M. and J.A.L. designed the study; S.M.M. performed the experiment, analyzed the data, and
wrote the initial manuscript draft; S.M.M. and J.A.L. contributed to revisions of the manuscript.

**Data accessibility** 

400 Data available from the Dryad Digital Repository: <u>https://doi.org/10.5061/dryad.ttdz08ktk</u>

401 (Magnoli and Lau, 2020)

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543 Figure 1 Mean plant biomass (estimated marginal mean±SE) of Lux, Marshall, and original 544 source plants inoculated with rhizobia from the Lux or Marshall site or no rhizobia, grown under 545 lower and higher nitrogen conditions. Statistical significance is indicated as follows:  $^+P < 0.08$ , \**P*<0.05, \*\**P*<0.01. 546



**Figure 2** Rhizobium effects shown as the log response ratio (ln biomass with rhizobia – ln biomass without rhizobia) on total plant biomass under lower and higher nitrogen conditions. Statistical significance is based on pairwise post-hoc contrasts comparing a population's mean biomass when inoculated with rhizobia to its mean biomass when un-inoculated using the dunnettx method for p-value adjustment, and is indicated as follows:  $^+P<0.08$ ,  $^*P<0.05$ ,  $^{**}P<0.01$ .



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**Figure 3** Mean nodule number (estimated marginal means±SE) and estimated mean nodule mass produced by plants (a,c) inoculated with rhizobia from the Lux or Marshall site and (b,d) grown under lower or higher nitrogen conditions, Plants inoculated with rhizobia from the Lux site produced more, heavier nodules than those inoculated with rhizobia from the Marshall site, and those grown under low nitrogen conditions tended to produce more, but not heavier, nodules than those grown under high nitrogen conditions.

