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Diversity of Nitrogen-Fixing Symbionts of *Chamaecrista fasciculata* (Partridge Pea) Across Variable Soils

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Abstract - We evaluated whether geographic distance and soil characteristics influence genetic structure of nitrogen-fixing bacterial symbionts associated with the host plant *Chamaecrista fasciculata* (Partridge Pea). We tested phylogeographic clustering and associations between genetic distance, geographic distance, and soil variables using sequences of 2 bacterial genes and soil chemistry across 23 sites in Mississippi. We identified rhizobia isolated from Partridge Pea as *Bradyrhizobium*. We detected significant genetic structure at a regional level, and determined that rhizobia within each region were more phylogenetically related than expected. Significant correlation between genetic distance and distances based on soil chemistry suggests environmental influences on rhizobia diversity. High levels of diversity among rhizobia over small spatial scales suggest that symbionts respond to local factors. Understanding geographic diversity in natural assemblages of rhizobia aids in predicting how hosts and symbionts respond to environmental perturbations.

Introduction

Approximately 88% of legume species are known to form symbiotic relationships with nitrogen-fixing bacteria known as rhizobia (Graham and Vance 2003). The great diversity of legume taxa, estimated at 20,000 species (Cronk et al. 2006), coupled with their presence in many different habitats around the world (Dolye and Luckow 2003, Yahara et al. 2013), may indicate that symbioses with rhizobia have contributed to promoting diversification across this family (Martínez-Romero and Caballero-Mellado 1996, Sprent 2009). Béna et al. (2005) showed that some *Medicago* (medick) species capable of forming symbioses with multiple rhizobia strains have larger ranges, yet other medicks evolved toward highly specialized relationships with few rhizobia as a result of lower fitness associated with hosting numerous symbionts. Characterization of diversity of rhizobia within and across host species in natural ecosystems is key to understanding the influence of rhizobia on legume diversification and the impact of such symbioses on ecosystem functioning historically and in the face of climate and land-use changes.

Numerous studies of microbial communities have found significant geographic structure in diverse environments and across diverse bacterial taxa (O'Malley 2008, Papke et al. 2003, Rout and Callaway 2012, Staley and Gosink 1999, Whitaker et al. 2003). The composition of free-living soil bacterial communities can be due to abiotic factors (Pasternak et al. 2013, Xiong et al. 2012) as well as

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impacts from other soil organisms (Djigal et al. 2004, Toljander et al. 2007) and plants, particularly through root exudates (Marschner et al. 2004). Among abiotic factors, climate, precipitation, organic matter, and soil texture have been found to influence soil microbial biogeographic patterns, but the most prominent variable influencing soil microbial diversity may be soil pH (Chong et al. 2012, Fierer and Jackson 2006, Griffiths et al. 2011, Lauber et al. 2008).

Rhizobia have intimate relationships with their plant hosts; thus, biogeographic patterns of symbiotic rhizobia may reflect selection by host species at the centers of origin and associated diversification with hosts across landscapes (Martínez-Romero and Caballero-Mellado 1996). As new legume species evolve and species expand their distributions, plants may maintain original relationships with symbionts if rhizobia are widespread, or they may evolve the ability to recognize and associate with new rhizobia types. While rhizobia and legume hosts may not be strictly co-evolving, plant hosts could influence biogeographic patterns of rhizobia by selecting for certain genotypes. For example, Sachs et al. (2009) found that plant-host identity significantly explained nodulating rhizobia diversity, as host plants were infected by a small subset of rhizobia available in the soil. Thus, geographic structure in symbiotic rhizobia may also be expected, but the relative strength of environment versus host species in determining biogeographic patterns of the symbionts remains understudied across diverse host species and ecosystems.

In this study, we characterized genetic diversity of rhizobia symbionts of a common and widespread legume, *Chamaecrista fasciculata* (Michx.) Greene (Partridge Pea), which is an important species in many natural ecosystems because it provides cover, nectar, and pollen for animals. It has also been of interest in agricultural systems, for example in crop rotation to enhance soil nitrogen (Reeves 1994) and to manage root-knot nematodes (Rodríguez-Kábana et al. 1995). Given its annual habitat, herbaceous growth form, and phylogenetic position as one of the only nodulating Caesalpinioids, there is growing interest in developing Partridge Pea as a model for studies of legume evolution (Singer et al. 2009). Partridge Pea has a geographic range that extends from Minnesota to the Gulf of Mexico and from the east coast of the US to New Mexico (USDA–NRCS 2015). Plants grow in open habitats such as prairies, bluffs, riverbanks, and upland woods, and can grow in a variety of soils (Pullen 1963, USDA–NRCS 2015). Phenotypic differences in Partridge Pea have been noted in plant morphology at local scales (e.g., Pullen 1963; L. Wallace, pers. observ.; Weakley 2012) and among widely separated populations (e.g., Galloway and Fenster 2000, Henson et al. 2013), suggesting great potential for locally adapted populations. The morphological and ecological variation exhibited by this species has been recognized by some taxonomists to represent distinct taxa (e.g., Weakley 2012), but these have not yet been shown to be genetically differentiated or reproductively isolated. Whether geographic structure extends to rhizobia symbionts is unclear. Thus, in this study, we tested 2 hypotheses: (1) nodulating rhizobia of Partridge Pea are geographically structured, and (2) geographic structure is associated with variation in soil composition. We expected phylogenetic clustering of rhizobia strains by their physiographic region, which are partially defined by soil type. Furthermore, we expected

that soil pH would be associated with rhizobia diversity, as pH often shapes the structure of free-living microbial communities in soils (Fierer and Jackson 2006). We characterized phylogenetic diversity using a housekeeping gene and a gene on the symbiosis island to evaluate whether horizontal gene exchange (HGE) may influence local community structure of the rhizobia, as reported for other symbiotic *Bradyrhizobium* (Parker 2012, Parker and Rousteau 2014).

Field-site Description

We selected sites across the physiographic regions of Mississippi (Fig. 1) to represent variation in soil habitat. The sampling design was intended to capture variation in nodulating rhizobia at a regional geographic scale reflecting variation in plant communities and soils, rather than to characterize complete rhizobia diversity at individual sites or across the entire range of the host species. We sampled rhizobia in nodules of Partridge Pea from June to July 2013 from a total of 23 locations in the Blackbelt Prairie (n = 4), Tombigbee Hills (n = 3), North-Central Hills (n = 3), Loess Hills (n = 3), Delta (n = 3), Jackson Prairie (n = 3), and South-Central Hills (n = 4) (Table 1; Fig. 1; see Table S1 in Supplemental File 1, available online at http://www.eaglehill.us/SENAonline/suppl-files/s18-1-S2494-Wallace-s1, and for BioOne subscribers, at https://dx.doi.org/10.1656/S2494.s1).

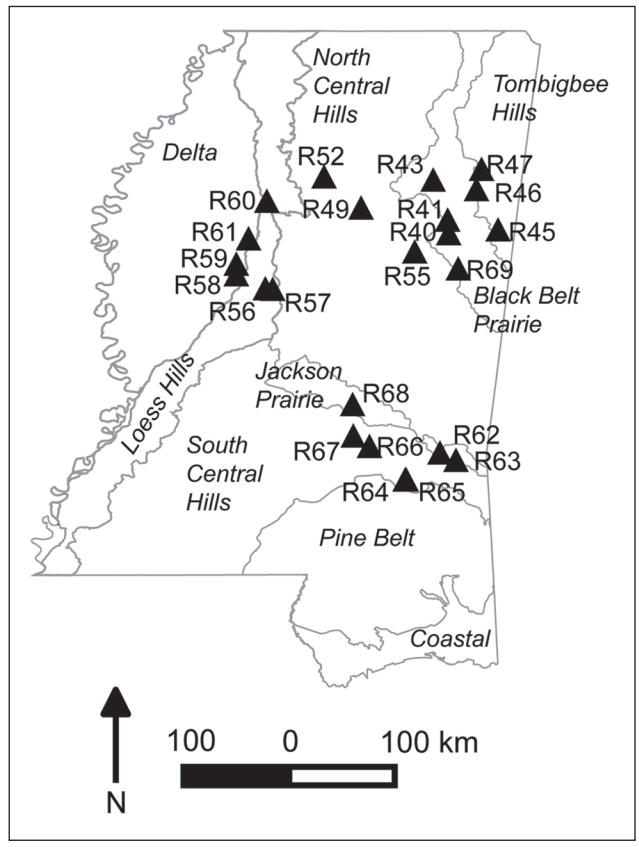

Figure 1. Sample locations of Partridge Pea within the designated physiographic regions of Mississippi.

We excavated roots from individual flowering plants randomly selected at each site across the area of growth of the species. We did not quantify or consider plant size or herbivory when selecting plants to sample. Sampling continued until we had collected roots with at least 1 nodule from a minimum of 12 plants per site; we did not sample plants growing immediately adjacent to a sampled plant. We placed each sampled root with its nodule(s) in a distinct bag to reduce contamination by bacteria from other plants. At each sampling location, we collected a voucher-plant specimen, soil sample from the top 25 cm within an area of extant host plants, and GPS coordinates (see Table S1 in Supplemental File 1, available online at http://www.eaglehill.us/SENAonline/suppl-files/s18-1-S2494-Wallace-s1, and for BioOne subscribers, at https://dx.doi.org/10.1656/S2494.s1). We deposited plant vouchers in the Mississippi State University (MISSA) herbarium (Mississippi State, MS). Upon return to the laboratory, we stored roots with nodules at -80 °C until processing for DNA extraction.

Methods

Genetic analysis

We were unable to obtain high-quality DNA or sequence data for some sampled plants. Thus, the final data-set included sequences collected from 117 distinct plants and 183 nodules (see Table 1 for sample sizes in each physiographic region). Rhizobia DNA from 1 or more nodules for 6 plants per collection site were extracted and diluted in 200 µl buffer using the Qiagen DNeasy plant Mini Kit (Qiagen, Valencia, CA). Prior to grinding, we surface-sterilized nodule samples in a 1% bleach solution for 5 min and then washed them in sterile water for 5 min. We placed nodules in 70% ethanol for 5 min, followed by a final 5-min sterile-water wash. We characterized rhizobia diversity across the sample sites based on partial

Table 1. Genetic diversity of *Bradyrhizobium* species across physiographic regions of Mississippi. *n* = number of clean, readable sequences generated; S = segregating sites; H = number of unique sequences; Hd = haplotype diversity; π = nucleotide diversity; BBP = Blackbelt Prairie; TH = Tombigbee Hills; NCH = North-Central Hills; LH = Loess Hills; D = Delta; JP = Jackson Prairie; and SCH = South-Central Hills.

	BBP	TH	NCH	LH	D	JP	SCH
truA							
n	36	38	29	17	18	13	32
S	123	173	87	116	96	73	89
H	19	25	12	9	12	9	19
Hd	0.954	0.950	0.894	0.831	0.948	0.936	0.944
π	0.1235	0.1103	0.0543	0.0817	0.1193	0.1127	0.0846
nifH							
n	35	28	27	16	18	10	27
S	113	101	86	70	46	67	53
H	19	16	14	8	8	7	12
Hd	0.923	0.947	0.875	0.808	0.752	0.867	0.872
π	0.0757	0.0775	0.0270	0.0493	0.0513	0.0484	0.0576

sequences of a symbiosis-island gene, *nifH*, and a housekeeping gene, *truA*. The *nifH* gene is involved in nitrogen fixation (Laguerre et al. 2001), and the *truA* gene is involved in translation and ribosomal biogenesis (Ahn et al. 2004). Both of these markers have been used by others to characterize rhizobia diversity (Vinuesa et al. 2005, Zhang et al. 2012). We amplified and sequenced *nifH* using primers outlined in Vinuesa et al. (2005), whereas *truA* was amplified and sequenced using primers from Zhang et al. (2012). We employed PCR to amplify the regions in 12.5-µl volumes containing 1.5 µl DNA, 1x LongAmp buffer (New England Biolabs, Ipswich, MA), 0.8% DMSO, 1.5 U LongAmp Taq (New England Biolabs), 0.32 mM dNTPs, 0.4 µM forward primer, and 0.4 µM reverse primer. For both genes, we heated the reaction tubes to 95 °C prior to the addition of DNA. The *nifH* program consisted of denaturation at 95 ºC for 3.5 min, 30 cycles of 93.5 ºC for 1 min, 58 ºC for 1 min, 72 ºC for 1 min, and an elongation step of 72 ºC for 5 min. *truA* required a touchdown thermal-cycler program as follows: denaturation at 95 ºC for 5 min, 11 cycles of 94 ºC for 45 sec, 60 ºC for 1 min decreased by 1.0 ºC per cycle, 72 ºC for 1 min, 26 cycles of 94 ºC for 45 sec, 50 ºC for 1 min, 72 ºC for 1 min, and an elongation step of 72 ºC for 10 min. We determined amplification of PCR products by agarose gel electrophoresis and ethidium bromide staining. We included a negative control with each set of reactions to check for contamination. We cleaned PCR products using 0.2x Antarctic Phosphatase buffer (New England Biolabs), 5 units Exonuclease I (New England Biolabs), and 1.25 units Antarctic Phosphatase followed by cycle sequencing in 10-µl reactions using forward and reverse primers and Big Dye version 3.1 (Life Technologies, Carlsbad, CA). We dried and sent sequenced samples to Arizona State University DNA Lab for capillary electrophoresis. Forward and reverse sequences were edited and assembled into a consensus sequence using Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, MI). We employed the AUTO option in MAFFT (Katoh et al. 2005) provided by the Computational Biology Research Center (http://mafft.cbrc.jp/alignment/server/) to align sequences. We deposited sequences in GenBank (NCBI 1988) as accessions KR186321–KR186443.

We determined sequence variation for each gene in each of the 7 geographic regions using DNAsp v. 5 (Librado and Rozas 2009) by calculating number of variable sites (S), number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (π). We conducted analyses of molecular variance (AMOVA; Excoffier et al. 1992) to quantify the distribution of variation within and across geographic regions for each gene; analyses were performed in Arlequin v. 3.5 (Excoffier and Lischer 2010) using pairwise distance of DNA sequences and designating sequences by physiographic region of origin. We generated phylogenetic trees for each of the genes to assess evolutionary relatedness of the strains. We downloaded reference sequences of taxonomically valid *Bradyrhizobium* taxa from GenBank (NCBI 1988) for use in the analyses to place the newly collected sequences in the broader context of known *Bradyrhizobium* diversity. For *truA,* the reference sequences included *B. canariense* (BTA1), *B. elkanii* (USDA 76), *B. japonicum* (USDA 6), *B. liaoningense* (USDA 3622), and *B. yuanmingense* (CCBAU 10071). For *nifH*,

reference sequences included *B. canariense* (BES1, BC-C2), *B. elkanii* (USDA 46, USDA 94), *B. japonicum* (DSMZ 30131, USDA 122, X3-1, X6-9, Nep1, Blup-MR1, FN13), *B. liaoningense* (LMG 18230, Spr 3-7), *B. yuanmingense* (CCBAU 10071), and unidentified *Bradyrhizobium* isolated from Partridge Pea (cf1, cf4, cfhr1a, cfrr1) or *C. nictitans* (L.) Moench (Sensitive Patridge Pea) (cnw15) by Parker (2012). We subjected each aligned data set to an assessment by jModeltest2 (Darriba et al. 2012, Guindon and Gascuel 2003) to determine the best-fitting model of molecular evolution under the BIC. We conducted these analyses using the Cipres Science Gateway (Miller et al. 2010). We selected TrN+I+G (Tamura and Nei 1993) as the best model for the *nifH* data set, and HKY+G (Hasegawa et al. 1985) as the best model for the *truA* sequences. We implemented GTR+I+G (Tavaré 1986), the closest model to TrN+I+G that can be used in MrBayes, for *nifH* and HKY+G for *truA* in independent Bayesian phylogenetic analyses using MrBayes v. 3.2.3 (Ronquist et al. 2012) in the Cipres Science Gateway (Miller et al. 2010). For each analysis, we conducted Markov Chain Monte Carlo (MCMC) for 5 million generations with a sampling frequency of 1000, after which the split standard deviation was less than 0.006. We set a burn-in of 1250 trees prior to determining the posterior probability of the trees with the highest likelihood. Consensus trees are reported with posterior probability indicated as support for clades. We used Phylocom 4.0.1 (Webb et al. 2008) to evaluate if rhizobia sequences were phylogenetically clustered by physiographic region. For each region, we calculated mean phylogenetic distance (MPD_{sample} ; Webb et al. 2008) across all pairs of bacterial sequences, and determined significance by comparing MPD_{sample} to a null distribution inferred from 1000 random permutations of sequences across the tips of the tree. We calculated a standardized measure of clustering across unequal sample sizes, the net relatedness index (NRI), as the difference between MPD_{sample} and the MPD_{null}, divided by the standard deviation of MPD_{null} (Webb et al. 2008). We conducted these analyses independently on the data sets.

Soil analysis

Soil samples were allowed to air dry for 20 d before we ground them using a mortar and pestle. We removed any visible organic fragments >5 mm prior to grinding. We sent the soil samples to the University of Arkansas for analysis of pH, nitrate (NO_3), ammonium (NH_4), phosphorus, potassium, magnesium, sulfur, sodium, iron, manganese, zinc, copper, and boron. We used these variables to assess possible soil factors affecting rhizobia assemblages. We employed Spearman's non-parametric correlation (Spearman 1907) to identify redundant soil variables in the dataset based on a correlation coefficient of 0.6 or higher between any 2 variables. We conducted correlation analyses in SPSS v. 21 (IBM Corporation 2012). Boron and magnesium exhibited significant correlation coefficients greater than 0.6 with several other variables; thus, we eliminated them from further analysis. Boron levels were correlated with levels of nitrate, calcium, zinc, and copper. Magnesium and potassium levels were correlated. We tested spatial autocorrelation of soils using a Mantel test (Mantel 1967) between a composite-soil distance and geographic

distance in PASSaGE 2 (Rosenberg and Anderson 2011). We generated the geographic-distance matrix using the linear distance between each sampling site and the haversine formula (Sinnott 1984). We used a squared Euclidian distance with a *Z*-score variance correlation in SPSS v. 21 (IBM Corporation 2012) to calculate a composite pairwise distance-matrix of sites based on all soil variables except boron and magnesium. We assessed significance of the correlation in the Mantel test with 999 permutations in a 1-tailed test, and defined significant correlations as $P < 0.05$. We also examined associations of soil properties and geography with genetic variability of rhizobia using partial Mantel tests (Smouse et al. 1986) in comparisons of: (1) genetic distance vs. a composite distance of soil variables while controlling for geographic distance, (2) genetic distance vs. distance based on soil pH while controlling for geographic distance, (3) genetic distance vs. geographic distance while controlling for soil characteristics. We tested soil pH separately from soil-mineral variables because previous studies suggested pH as the factor most significantly affecting soil bacterial assemblages (e.g., Fierer and Jackson 2006). We also calculated the matrix of soil pH distances in SPSS using squared Euclidean distance and *Z*-score variance. We determined pairwise genetic distance, calculated as p-distance, between sample sites using MEGA v. 6 (Tamura et al. 2013). We conducted Mantel tests for each genetic data set and the geographic- and environmental-distance matrixes independently using PASSaGE 2 (Rosenberg and Anderson 2011) as described above. We excluded site R63 from the Mantel tests due to the small number of sequences collected from this site.

Results

Genetic diversity and phylogenetic patterns

In total, we generated 161 *nifH* sequences and 183 *truA* sequences from rhizobia in nodules that were successfully sequenced. Sequences generated in this project most closely matched *Bradyrhizobium* sequences in BLAST-n searches with GenBank (NCBI 1988). The aligned lengths were 706 nucleotides for *nifH* (~69% coverage of the gene in *Bradyrhizobium*) and 497 nucleotides for *truA* (~67% coverage of the gene in *Bradyrhizobium*). We found high levels of genetic diversity in each of the data sets. Nucleotide diversity was higher in *truA* compared to *nifH* for all geographic regions. Haplotype diversity varied from 0.831 to 0.954 for *truA* and from 0.752 to 0.947 for *nifH*. Regions for which we obtained more samples exhibited a greater number of haplotypes and increased haplotype diversity in *nifH*, but differences in sampling intensity were not apparent in the number of segregating sites or nucleotide diversity for either gene or for haplotype diversity in *truA*. Diversity measures for each of the regions are reported in Table 1.

The diversity of *Bradyrhizobium* symbionts found in Mississippi encompasses multiple recognized species in this genus, as evidenced by the phylogenetic clustering of newly collected samples with reference sequences of *B. elkanii, B. japonicum, B. liaoningense,* and *B. yuanmingense* (Figs. 2, 3). The topologies of the phylogenies both depict 2 large, well-supported clades (labeled as A and B in Figs. 2, 3; posterior probability > 0.99), with several smaller clades and singleton

strains clustering external to these clades. The identities of clades A and B are relatively conserved between the data sets, but some samples are phylogenetically incongruent between the trees (see Figure S1 in Supplemental File 1, available online at http://www.eaglehill.us/SENAonline/suppl-files/s18-1-S2494-Wallace-s1, and for BioOne subscribers, at https://dx.doi.org/10.1656/S2494.s1).

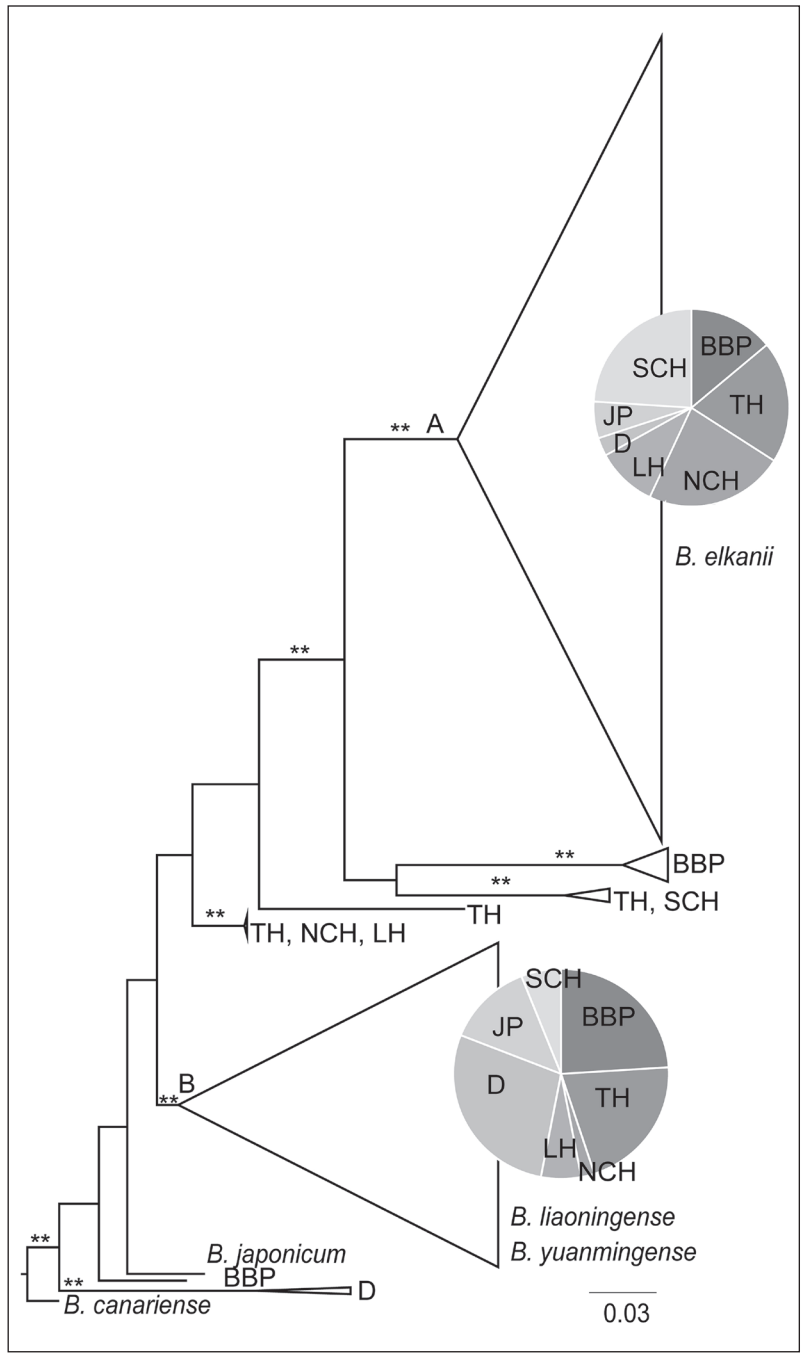

Figure 2. Phylogram of sampled *Bradyrhizobium* strains and reference sequences based on variation in *truA*. Strains have been grouped into clades that exhibit strong support. Samples of outgroups and related taxa downloaded from GenBank are indicated on the tree or beside the clade in which they clustered. Pie charts for clades A and B indicate proportional make-up from each of the designated physiographic regions as follows: BBP = Blackbelt Prairie; TH = Tombigbee Hills; NCH = North-Central Hills; LH = Loess Hills; D = Delta; JP = Jackson Prairie; SCH = South-Central Hills. Support values are indicated on branches by asterisks (** >95% posterior probability).

The majority of hosts with multiple nodules exhibited rhizobia sequences that were less than 10% divergent, although some plants did contain divergent strains. Considering greater than 6% difference in *truA* to signal interspecific differences (based on the assessment by Zhang et al. [2012] that included *truA* sequences), we identified 23 plants in our data set that contained highly divergent rhizobia reflecting species-level differences among *Bradyrhizobium*. Considering greater than 5% difference as a signal of species-level differences in *nifH* (Gaby and Buckley 2012), we identified 11 plants containing different species of symbionts. Eight plants

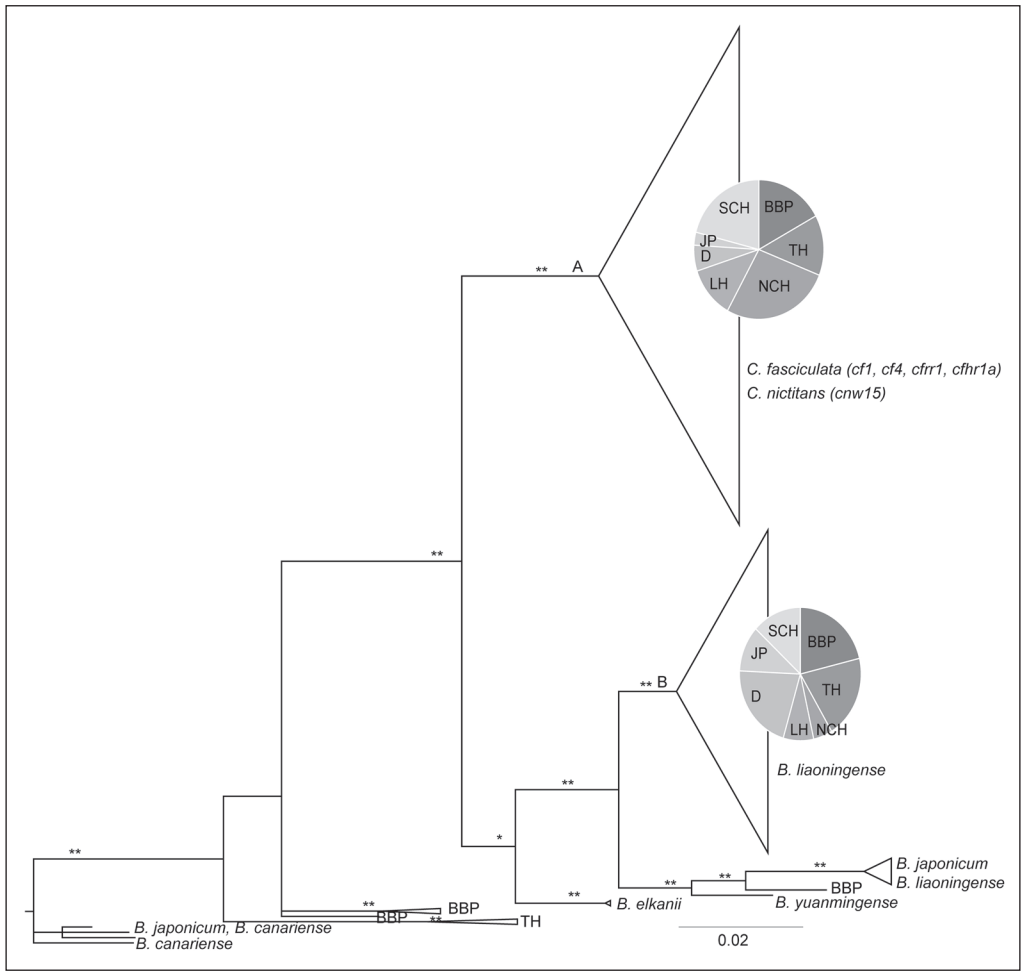

Figure 3. Phylogram of sampled *Bradyrhizobium* strains and reference sequences based on variation in *nifH*. Strains have been grouped into clades that exhibit strong support. Samples of outgroups and related taxa downloaded from GenBank are indicated on the tree or beside the clade that they clustered in. Pie charts for clades A and B indicate proportional make-up from each of the designated physiographic regions as follows: BBP = Blackbelt Prairie, TH = Tombigbee Hills, NCH = North-Central Hills, LH = Loess Hills, D = Delta, JP = Jackson Prairie, SCH = South-Central Hills. Support values are indicated on branches by asterisks (** >95% posterior probability; * > 90% posterior probability).

exhibited concordance between *truA* and *nifH* data sets in having highly divergent symbionts between nodules. These were distributed across 7 sample sites.

Analyses of sequence and phylogenetic diversity detected geographic structure in the symbionts of Partridge Pea. AMOVA for both genes indicated significant molecular variation among the defined physiographic regions (*truA* F_{ST} = 0.134, $P < 0.001$; *nifH* F_{ST} = 0.123, $P < 0.001$; Table 2). Strains also exhibited significant phylogenetic clustering for 4 of the 7 physiographic regions in the *truA* dataset and for 5 of the regions in the *nifH* data set (Table 3). When we observed significant patterns, rhizobia contained gene variants that were more similar, on average, compared to the null distribution of permuted sequences across the trees for all cases except *nifH* in the Blackbelt Prairie. For this region, *nifH* gene variants were significantly over-dispersed across the phylogeny relative to the null distribution.

Comparisons of genetic and soil variation

Soils were highly variable across sampling sites, and the correlation between geographic distance and the composite soil-distance was weak (r = -0.018, t = -0.205, $P > 0.05$; Table 4), although sites are distinguishable in canonical discriminant analysis (see Figure S2 in Supplemental File 1, available online at http://www.eaglehill.us/SENAonline/suppl-files/s18-1-S2494-Wallace-s1, and for BioOne

Table 2. Analysis of molecular variance (AMOVA) of *Bradyrhizobium* genotypes determined by *truA* and *nifH* sequences across the physiographic regions of Mississippi (see Fig. 1). DF = degrees of freedom.

	DF	Sum of squares	Variance component	Percent variation	Fixation index
truA					
Among regions	6	556.801	2.896	13.42	F_{ST} = 0.134; $P < 0.001$
Within regions	176	3289.270	18.689	86.58	
Total	182	3846.071	21.585	100.00	
nifH					
Among regions	6	348.832	1.960	12.32	F_{ST} = 0.123; $P < 0.001$
Within regions	154	2148.367	13.950	87.68	
Total	160	2497.199	15.911	100.00	

Table 3. Phylogenetic clustering of *Bradyrhizobium* symbionts in relation to physiographic region. n = sample size, NRI = net relatedness index, and P = level of significance. Asterisks (*) indicate significant correlations ($P < 0.05$).

Region	*truA n*	NRI	*P*	*nifH n*	NRI	*P*
Blackbelt Prairie	36	-1.1276	0.112	35	-2.243	0.009*
Tombigbee Hills	38	1.7639	0.048*	28	-0.401	0.349
North-Central Hills	29	7.1819	<0.001*	27	5.614	<0.001*
Loess Hills	17	1.7079	0.063	16	1.974	0.012*
Delta	18	1.9522	0.042*	18	2.729	0.005*
Jackson Prairie	13	0.5532	0.233	10	1.177	0.100
South-Central Hills	32	4.9874	<0.001*	27	2.757	0.004*
All	183	3.7790	<0.001*	161	3.174	0.003*

subscribers, at https://dx.doi.org/10.1656/S2494.s1). Many sites within the Blackbelt Prairie contained high levels of calcium, which is characteristic of the white soils common to this area. Many sites in the Delta, which is highly agricultural, contained higher levels of phosphorus and potassium. In contrast to a lack of autocorrelation in soil variables, we identified significant associations between genetic distance and distance based on soil properties. When controlling for geographic distance, genetic distance was correlated with soil distance for both genes (*truA:* $r = 0.413$, $t = 2.465$, $P = 0.01$; *nifH:* $r = 0.303$, $t = 2.047$, $P = 0.049$) as well as distance based solely on pH (*truA:* $r = 0.202$, $t = 1.783$, $P = 0.04$; *nifH:* $r = 0.366$, $t = 3.550$, $P = 0.001$). Genetic distance was not correlated with geographic distance when controlling for variation in soil characteristics for either gene (*truA* $r = -0.082$, $t = -0.971$, $P = 0.20$; *nifH* $r = -0.048$, $t = -0.591$, $P = 0.320$).

Discussion

Genetic diversity in relation to geography and soils

In this study, we found that rhizobia symbionts of the host plant Partridge Pea exhibited high levels of genetic diversity and geographically relevant structure. Our results substantially extend understanding of the diversity of native rhizobia symbionts associated with Partridge Pea and in ecosystems that have not been examined previously (Koppell and Parker 2012, Parker 2015, Parker and Kennedy 2006). All sequences generated in this study are most similar to other *Bradyrhizobium* strains, a finding that is consistent with the exclusive use of *Bradyrhizobium* by *Chamaecrista* (sensitive pea) host plants (Andrews and Andrews 2017). Many *Bradyrhizobium* species are considered to be generalists because they nodulate wild legume species from different genera (Ehinger et al. 2014, Koppell and Parker 2012) and some agriculturally important species that are widely planted (Appunu et al. 2008). Our results mirror other studies that have reported diverse symbiotic partners in widespread legume hosts, including *Medicago sativa* L. (Alfalfa) (Paffetti et al. 1996), *Vicia faba* L. (Broad Bean) (Tian et al. 2007), and *Acacia pycnantha* Benth. (Golden Wattle) (Ndlovu et al. 2013). For many of these species, it has been suggested that enhanced diversity of symbionts may underlie the success of host plants in non-native habitats (e.g., Ndlovu et al. 2013) and influence the evolution

Table 4. Mantel and partial Mantel tests of matrix correlations between distances based on genetic, geographic, soil, and soil pH variables. *r* = correlation; *t* = *t*-statistic; *P* = significance level. Asterisks (*) indicate significant correlations.

Comparison	*r*	*t*	*P*			
Soil vs. geographic	-0.018	-0.205	0.47			
	truA			*nifH*		
Comparison	*r*	*t*	*P*	*r*	*t*	*P*
Genetic vs. geographic, soil constant	-0.082	-0.971	0.20	-0.048	-0.591	0.320
Genetic vs. soil, geography constant	0.413	2.465	0.01*	0.303	2.047	0.049*
Genetic vs. soil pH, geography constant	0.202	1.783	0.04*	0.366	3.550	0.001*

of legume species (Zahran 2001). For Partridge Pea, we also found that symbionts can be highly diverse among nodules on an individual plant. As many as 23 plants in this study (~20%) contained species-level differences of *Bradyrhizobium* symbionts in their nodules. Intra-plant variation in rhizobia has been reported for other widespread legume hosts, including *Trifolium pratense* L. (Red Clover) (Hagen and Hamrick 1996). Given that legume success is highly dependent on the functionality of specific rhizobia genotypes through genotype x genotype interactions (Heath 2010), the ability to utilize diverse strains may underlie this host plant's ability to maintain such a wide geographic range across the eastern US.

It is now well known that free-living and symbiotic soil microbes in diverse geographic areas frequently exhibit genetic structure (O'Malley 2008, Papke et al. 2003, Rout and Callaway 2012, Staley and Gosink 1999, Whitaker et al. 2003), but the causes underlying this structure are not well understood. We also hypothesized that rhizobia symbionts of Partridge Pea would be geographically structured and that soil variables may underlie these patterns of distribution. In our study, analyses of molecular variance by region indicated that a significant proportion of the observed variation is distributed among the regions (Table 2). Nevertheless, we did not find a significant correlation between geographic and genetic distance in the Mantel test, indicating that the symbionts do not follow a strict isolation by distance pattern. This finding is consistent with other studies demonstrating that genetic structure of soil bacteria is largely independent of geographic distance (Fierer and Jackson 2006). When examined by phylogenetic relatedness, our Phylocom analyses indicated significant clustering of strains for both genes in the North-Central Hills, Delta, and South-Central Hills, which supports the results of AMOVA. Only Jackson Prairie showed non-significant values for both genes in these analyses. This result may be due to the small sample size for this region (10–13 sequences) compared to the other areas. Also noteworthy, the Blackbelt Prairie strains were more dispersed in both phylogenies than any other area, resulting in negative NRI values for both genes (Table 3). The Blackbelt Prairie contains a mixture of habitats, some with chalky, calcareous soils and others with rich dark soils. For this study, we did not characterize the type of soil where individual plants were growing, but note that we did sample in both soil types. Such a difference may underlie the reason for the negative NRI values and reflect a finer scale of structure among *Bradyrhizobium* symbionts if they (or their host plants) are locally adapted to very different soil types of this region.

Relative to other genera of rhizobia, *Bradyrhizobium* is considered to be a generalist group that is symbiotic with diverse host plants (Parker 2015). However, within this diverse genus there is evidence of host specificity and geographic localization of strains. Koppell and Parker (2012) identified superclades of *B. elkanii* and *B. japonicum* sampled from 41 legume genera from Alaska to Panama; individuals in these clades spanned all sampled regions, indicating little evidence of regional endemism at this deep phylogenetic level. At finer scales, *Bradyrhizobium* strains within the 2 superclades did exhibit geographical structure because distinct bacterial strains were restricted to geographically disparate areas (Koppell and Parker

2012). Our results provide support for geographic localization of strains at fine taxonomic and geographic scales. These results are also consistent with the larger-scale study by Koppell and Parker (2012) across multiple legume hosts and much of North America.

Given that a strict isolation-by-distance pattern was not satisfactory in explaining the phylogenetic structure of rhizobia isolated from Partridge Pea, other factors must be considered, including traits of the environment and plant host. Diversity and composition of soil microbial communities is often dictated by soil properties. Among these, pH has been found to have a strong effect on soil microbial communities (Chong et al. 2012, Fierer and Jackson 2006) because many bacteria are limited in their ability to survive in basic or acidic soils. For certain rhizobia, soil properties have also been found to influence their presence and diversity. For example, highly acidic soils show less rhizobia diversity than soils where the pH has been artificially increased with the addition of lime (Andrade et al. 2002). Multiple studies have identified differences in soil preferences between *Bradyrhizobium* and *Sinorhizobium* isolates and biogeographic patterns that are associated with soil pH (Li et al. 2011, Zhang et al. 2011) as well as available N, P, and K (Zhang et al. 2011). Soil pH and site elevation were found to correlate with diversity of *Mesorhizobium* symbionts (Lemaire et al. 2014). We identified a positive association between genetic distance and soil variation for Partridge Pea. The Mantel tests revealed significant positive correlations between the composite soil distance and rhizobia genetic distance for both genes, as well as distance based only on pH and genetic distance. Given that we found wide variation in soil minerals of sites considered to be in the same physiographic region, our sampling approach may not have captured the continuum of environmental variation to pinpoint specific variables of the soil and their relative influence on rhizobia diversity. Additionally, plant host could also influence symbiont structure because host plants can select their rhizobia partners (Hirsch et al. 2001) via signaling prior to the establishment of nodules (Yang et al. 2010). Sachs et al. (2009) found that the rhizobia housed in nodules were a subset of those in the surrounding soil, indicating a strong role for plant host to choose particular rhizobia genotypes. We did not characterize rhizobia in soil samples or host genotypes, thus these alternative factors cannot be fully evaluated in this study. Nevertheless, given the signal we identified between genetic distance and environmental variation, we expect that a combination of biotic and abiotic factors is likely to dictate biogeographic patterns of *Bradyrhizobium* symbionts. Additionally, given that Partridge Pea is an annual species, the diversity of symbiotic rhizobia may vary from year to year.

Horizontal gene exchange and symbiont diversity

In previous studies on diversity of *Bradyrhizobium* symbionts, genes located on the symbiosis island exhibited incongruent phylogenies when compared to those based on housekeeping genes (Koppell and Parker 2012, Parker 2012, Parker and Rousteau 2014, Parker et al. 2002,), suggesting strong potential for horizontal transfer of symbiosis genes among soil microbes. We did not find overwhelming evidence of incongruence between the phylogenies based on *nifH* and *truA* because

most of the samples clustered in 1 of 2 large and well-supported clades (Figs. 2, 3). For those samples that contained mismatched placements between the trees, acquisition of 1 of these genes may have occurred horizontally via exchange with other *Bradyrhizobium*. Nevertheless, both data sets show evidence of similar geographic structure. AMOVA indicated 12–13% of the variation is distributed among regions. There was also evidence of phylogenetic clustering in both genes for 3 of the physiographic regions, and Mantel tests indicated significant and positive correlations between matrixes of genetic distance and soil variables for both genes. Based on the similarities in phylogenetic signal of the 2 genes, we suggest that horizontal gene exchange is not likely a significant driver of the genetic structure observed in this study. The mismatched individuals occur across multiple sample sites and physiographic regions, which suggests the absence of local selection for particular variants. Although Parker and colleagues (Koppell and Parker 2012, Parker 2012, Parker and Rousteau 2014, Parker et al. 2002) found evidence consistent with horizontal gene-exchange among *Bradyrhizobium* at a broad geographic scale, they also found that most strains (as deduced from symbiosis genes) were associated with few host plants. Thus, within any given host plant–rhizobia symbiosis, there may be selection for maintenance of a more limited pool of compatible symbionts. Further studies of horizontal gene exchange in natural systems would help in understanding its importance in generating novel symbionts and their interactions with host plants. Partridge Pea has a wide distribution and occupies a diversity of habitats; thus, it is an ideal system in which to investigate the breadth of the effects of geography and environmental variables on the establishment of legume–rhizobia symbioses. Understanding natural assemblages of rhizobia associated with native legumes in the context of environmental heterogeneity will aid in predicting how hosts and symbionts are likely to respond to environmental perturbations.

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