AEM
Journals.ASM.org

Genetic Divergence of *Bradyrhizobium* Strains Nodulating Soybeans as Revealed by Multilocus Sequence Analysis of Genes Inside and Outside the Symbiosis Island

Xing Xing Zhang,[a,b,c] Hui Juan Guo,[a,b,c] Rui Wang,[a,b,c] Xin Hua Sui,[a,b,c] Yan Ming Zhang,[a,b,c] En Tao Wang,[d] Chang Fu Tian,[a,b,c] Wen Xin Chen[a,b,c]

State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, China[a]; Key Laboratory of Soil Microbiology, Ministry of Agriculture, China Agricultural University, Beijing, China[b]; Rhizobium Research Center, China Agricultural University, Beijing, China[c]; Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City, Mexico[d]

The genus *Bradyrhizobium* has been considered to be a taxonomically difficult group. In this study, phylogenetics and evolutionary genetics analyses were used to investigate divergence levels among *Bradyrhizobium* strains nodulating soybeans in China. Eleven genospecies were identified by sequence analysis of three phylogenetic and taxonomic markers (*SMc00019*, *thrA*, and *truA*). This was also supported by analyses of eight genes outside the symbiosis island ("off-island" genes; *SMc00019*, *thrA*, *truA*, *fabB*, *glyA*, *phyR*, *exoN*, and *hsfA*). However, seven genes inside the symbiosis island ("island" genes; *nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *trpD*, and *rhcC2*) showed contrasting lower levels of nucleotide diversity and recombination rates than did off-island genes. Island genes had significantly incongruent gene phylogenies compared to the species tree. Four phylogenetic clusters were observed in island genes, and the epidemic cluster IV (harbored by *Bradyrhizobium japonicum*, *Bradyrhizobium diazoefficiens*, *Bradyrhizobium huanghuaihaiense*, *Bradyrhizobium liaoningense*, *Bradyrhizobium daqingense*, *Bradyrhizobium* sp. I, *Bradyrhizobium* sp. III, and *Bradyrhizobium* sp. IV) was not found in *Bradyrhizobium yuanmingense*, *Bradyrhizobium* sp. II, or *Bradyrhizobium elkanii*. The gene flow level of island genes among genospecies is discussed in the context of the divergence level of off-island genes.

Soybeans (*Glycine max* L.) were first domesticated in China and then introduced into different parts of the planet (1), now with an annual harvest area of 100 million hectares around the world (FAO, 2011). Ninety percent of their production comes from the United States, Brazil, Argentina, China, and India (FAO, 2007 to 2011). One of the key features of soybean is its ability to form symbiotic nitrogen-fixing nodules with diverse rhizobial species (2, 3), implying its important role in sustainable agriculture. It has been recurrently reported that *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense*, and *Sinorhizobium fredii* could nodulate soybeans (2–5). Recently, *Bradyrhizobium huanghuaihaiense*, *Bradyrhizobium daqingense*, *Sinorhizobium sojae*, and several unnamed species were also found to be effective microsymbionts of soybeans (2, 3, 6–8). Strain USDA110 represents a widely distributed type formerly known as *B. japonicum* Ia, but it has recently been proposed as a member of the new species *Bradyrhizobium diazoefficiens* (9).

Recent studies not only suggested differences in the biogeographic distribution of rhizobial species nodulating soybeans but also demonstrated a biased selection of rhizobial species by different genotypes of soybeans (2, 4, 10, 11). Consistent with these findings, comparative genomics of rhizobia revealed that the phyletic distribution of rhizobial functional genes involved in environmental adaptations and symbiotic interactions generally agrees with the phylogeny of rhizobial species (7). Therefore, it is important to distinguish different rhizobial species and even subdivisions of each species. The genus *Bradyrhizobium* has been considered to be a taxonomically difficult group (12, 13). In contrast to the highly conserved *rrs* sequences in *Bradyrhizobium*, sequence analyses of housekeeping genes (*atpD*, *recA*, *glnII*, *gyrB*, *rpoB*, *dnaK*, etc.) have been found to be very useful in this scenario, especially when these genes were concatenated (5, 6, 8, 14–16). Although the phylogeny of the concatenated housekeeping genes was usually considered to represent the species tree, these housekeeping loci showed high rates of intergenic recombination and a limited gap between interspecies and intraspecies sequence similarities (15, 16). Recent developments in rhizobial genomics allowed us to construct a well-resolved, reliable species tree for rhizobia (7, 17). Then, three core genes, *SMc00019*, *truA*, and *thrA*, both alone and in combination, were found to be able to produce phylogenies supporting this predetermined species tree (17). Moreover, these core genes provide a gap of 2% for intraspecies/interspecies average nucleotide identity (ANI) for rhizobia, including *Bradyrhizobium* (17), implying their potential role as useful markers in taxonomy, phylogeny, and population genetics. In most studies on the phylogenetics and evolutionary genetics of housekeeping genes in *Bradyrhizobium*, no symbiosis genes were analyzed, or only *nifH* and/or *nodC* on the symbiosis island (5, 14–16, 18). On the other hand, studies on the evolution of nodu-

Received 6 January 2014 **Accepted** 7 March 2014
Published ahead of print 14 March 2014
Editor: S.-J. Liu
Address correspondence to Chang Fu Tian, changfutian@gmail.com.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00044-14.

doi:10.1128/AEM.00044-14

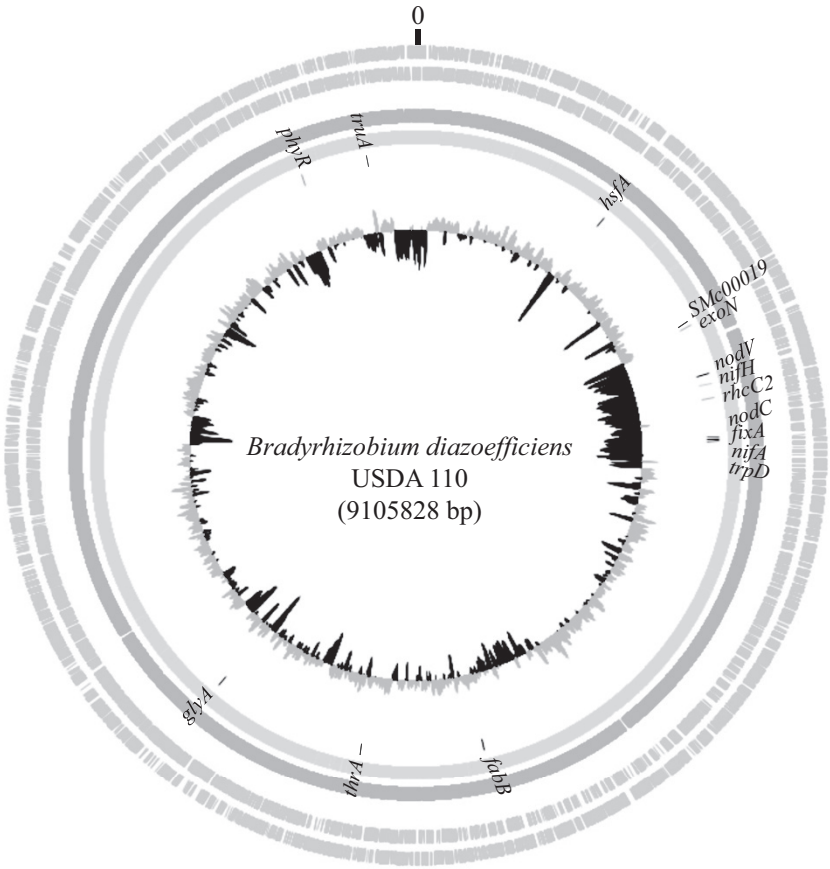


FIG 1 Locations of the genes used in this study on the genome of *Bradyrhizobium diazoefficiens* USDA110.

lation and nitrogen fixation genes were mainly focused on phylogenetics of these symbiosis genes (19–21).

In this study, we aimed at providing high-resolution delineations and evolutionary genetics analyses of *Bradyrhizobium* strains nodulating soybeans in China by studying seven genes (*nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *trpD*, and *rhcC2*) on the symbiosis island ("island" genes) and eight "off-island" genes (*SMc00019*, *thrA*, *truA*, *fabB*, *glyA*, *phyR*, *exoN*, and *hsfA*) (17, 22–32). Strains were assigned to genospecies based on the phylogeny and ANI values of core genes *SMc00019*, *truA*, and *thrA*. Molecular diversity, minimum recombination events, the topology of phylogenetic trees, and the levels of divergence and gene flow were compared between island genes and off-island genes.

MATERIALS AND METHODS

Bacterial strains. The 272 *Bradyrhizobium* strains used in this study (see Table S1 and Fig. S1 in the supplemental material) were previously collected from soybean nodules in four ecoregions of China (South China, Huanghuaihai, Northeast China, and Xinjiang) (2, 3, 6, 8, 10, 33). These strains were grown in TY medium at 28°C.

Primers, PCR amplifications, and sequencing. Total template DNA was extracted from each isolate using the GUTC method described by Terefework et al. (34). The PCR amplification of core genes *SMc00019*, *truA*, and *thrA* was performed with the procedure of Zhang et al. (17). The other 12 tested genes have been documented earlier (22–25, 28, 30, 31, 35–37) and were selected by considering their locations in the *B. diazoefficiens* USDA110 genome (Fig. 1). Briefly, *rhcC2*, *trpD*, *fixA*, *nifA*, *nifH*, *nodV*, and *nodC* are located within the symbiosis island, and *hsfA*, *exoN*, *phyR*, *glyA*, and *fabB* are outside the symbiosis island (Fig. 1). These 12 genes were amplified using primers designed in this study (see Table S2 in the supplemental material). All PCR products were commercially sequenced by BGI, China.

Phylogenetic analyses. Neighbor-joining trees were reconstructed using MEGA 5 (38). PHYML software (39) in combination with MODELTEST 3.7 (40) was used to build maximum likelihood (ML) trees. Moreover, the Shimodaira-Hasegawa (SH) test (41) was performed to investigate the global phylogenetic congruence of trees inferred from different sets of sequence partitions as implemented in PAUP (42).

Nucleotide polymorphism. DNASP V5 (43) was used to investigate nucleotide polymorphisms of test genes by calculating statistics as follows: the number of haplotypes (*h*), defined as sequence types (ST) for each gene and for the concatenated data, and the haplotype diversity (Hd) (44); the nucleotide diversity, π, defined as the average number of nucleotide differences per site between two sequences (44); and π_S, the nucleotide diversity for synonymous substitutions (d_S), and π_N, the nucleotide diversity for nonsynonymous substitutions (d_N) (45).

Evolutionary genetics analyses. The program CLUSTAL W integrated in MEGA 5 was used to align sequences (38). The "No. of differences" method integrated in MEGA 5 was used for calculating the pairwise distance between sequences of a single gene, from which ANI values were obtained using Excel (17). *Dxy*, the average nucleotide divergence between groups, and *Nm*, the number of migrants, were estimated by using DNASP (43, 44, 46). Minimal recombination events (*Rm*) at each locus or for the concatenated sequences were calculated and compared with the values expected under coalescence simulations based on 1,000 genealogy replications with DNASP (43, 47). CLONALFRAME was used to calculate

TABLE 1 Accession numbers of sequences obtained in this study

Gene	Accession numbers	Gene function	Reference
SMc00019	KF473160–KF473235	Conserved hypothetical protein	17
truA	KF473388–KF473463	tRNA pseudouridine synthase A	17
thrA	KF473236–KF473311, KF988139–KF988159, KF988162–KF988336	Homoserine dehydrogenase	17
hsfA	KF472552–KF472627	Host-specific nitrogen fixation	22
exoN	KF472331–KF472406	UTP-glucose-1-phosphate uridylyltransferase	65
phyR	KF473008–KF473083	Two-component response regulator	23
glyA	KF472476–KF472551	Glycine hydroxymethyltransferase	24
fabB	KF472856–KF472931	Beta-ketoacyl acyl carrier protein synthase	27
rhcC2	KF473084–KF473159	Type III secretion system component	32
trpD	KF473312–KF473387	Anthranilate phosphoribosyltransferase	55
fixA	KF472407–KF472475, KJ551550–KJ551556	Electron transfer flavoprotein FixA	25
nifA	KF472628–KF472703	Transcriptional regulator for nitrogen fixation genes	28
nifH	KF472704–KF472779	Nitrogenase Fe protein	25
nodV	KF472932–KF473007	Two-component regulator	30
nodC	KF472780–KF472855	*N*-Acetylglucosaminyltransferase	29

ρ/θ (the relative frequency of the occurrence of recombination compared with point mutation in the history of the lineage) and *r/m* (the relative impact of recombination compared with point mutation in the genetic diversification of the lineage) as described earlier (48, 49).

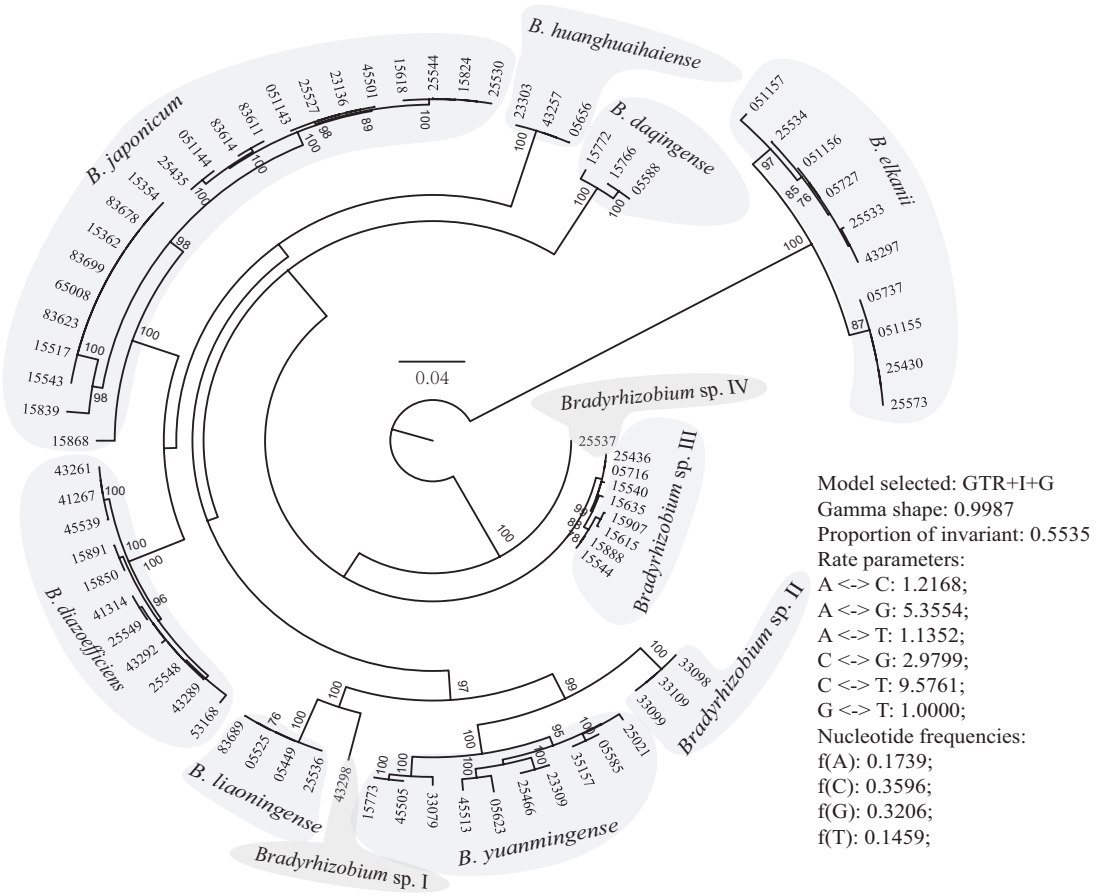
Nucleotide sequence accession numbers. The 1,336 nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers KF472331 to KF473463, KJ551550 to KJ551556, KF988139 to KF988159, and KF988162 to KF988336 (Table 1).

RESULTS AND DISCUSSION

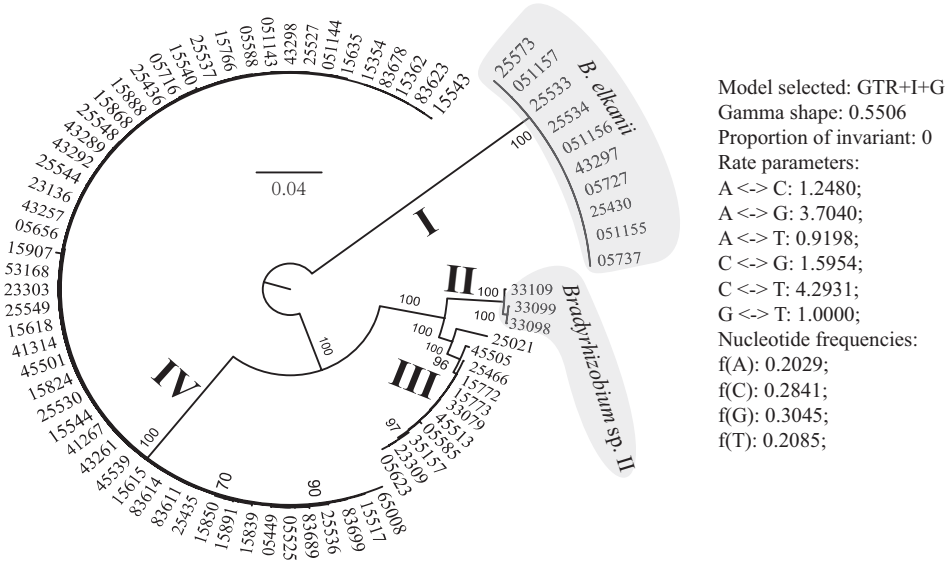
Eleven genospecies of *Bradyrhizobium* nodulate soybeans in China. China is considered to be the domestication center of soybeans and harbors the highest known diversity of rhizobia nodulating soybeans (1–6, 8, 10, 18, 33). However, only a few strains of this rhizobial germplasm were included in an earlier evolutionary genetics study of *Bradyrhizobium* nodulating soybeans (5). In this study, 272 strains from our earlier studies of soybean rhizobia in four ecoregions of China (see Fig. S1 and Table S1 in the supplemental material) and type strains of related *Bradyrhizobium* species were subjected to sequence analyses of *thrA*, a useful phylogenetic and taxonomic marker for rhizobia (17). Based on an intraspecies boundary of 96% ANI (17), and phylogenetic relationships among strains, 9 genospecies were identified: *B. japonicum* (50 strains), *B. diazoefficiens* (46 strains), *B. elkanii* (12 strains), *B. yuanmingense* (27 strains), *B. liaoningense* (31 strains), *B. daqingense* (26 strains), *B. huanghuaihaiense* (32 strains), *Bradyrhizobium* sp. I (1 strain), and *Bradyrhizobium* sp. III (41 strains). For strains with identical *thrA* sequences, representatives were selected for sequencing the other 14 test genes by considering their geographic origin. Finally, the PCR products of 15 tested loci were successfully obtained for 76 out of 81 representative strains. Therefore, these 76 strains were used in further analyses (see Table S3 in the supplemental material).

In the ANI analysis of the *SMc00019-truA-thrA* concatenate, 72/76 test strains were grouped into the same nine species according to the 96% intraspecies boundary (17). They are *B. japonicum* (containing 22 isolates), *B. diazoefficiens* (11 isolates), *B. daqingense* (3 isolates), *B. elkanii* (10 isolates), *B. huanghuaihaiense* (3 isolates), *B. liaoningense* (4 isolates), *B. yuanmingense* (10 isolates), *Bradyrhizobium* sp. III (8 isolates), and *Bradyrhizobium* sp. I (CCBAU 43298). CCBAU 33098, 33099, and 33109 showed a maximum ANI value of 95.6% with the other test genospecies in the *SMc00019-truA-thrA* concatenate and were consequently considered distinct and named *Bradyrhizobium* sp. II. CCBAU 25537 had a maximum ANI value of 93.8% with the other genospecies and was named *Bradyrhizobium* sp. IV. The assignments to the known *Bradyrhizobium* species were also supported by the well-resolved maximum likelihood tree of the *SMc00019-truA-thrA* concatenate for the tested strains and related type strains of the corresponding *Bradyrhizobium* species (see Fig. S2 in the supplemental material), as well as that of all the off-island core genes (Fig. 2a). Out of the 76 strains, 66 were classified into the same species as described in previous studies (2, 3, 10, 33). Eight out of 10 of the strains with a discrepancy in species assignment had previously been classified using only restriction fragment length polymorphism of the 16S-23S rRNA gene intergenic spacer region (IGS-RFLP) or BOX-A1R primer-based repetitive extragenic palindromic PCR (BOX-PCR) clustering. In an earlier study (3), CCBAU 05716 might have been improperly classified into *B. japonicum* considering its relatively low similarity values with other species in *atpD-glnII-recA* sequences (<93.4%). CCBAU 33109 was previously identified as *B. yuanmingense* based on its sequence similarity values of *atpD* (97.8%), *glnII* (95.8%), and *recA* (97.1%) with the type strain CCBAU 10071^T of *B. yuanmingense* (10) but was defined as *Bradyrhizobium* sp. II in this study by using *SMc00019-truA-thrA* sequences (ANI <95.64% with other species). So, the sequence-based classification has a great advantage over electrophoresis patterns in terms of data sharing and reinvestigations. Among these *Bradyrhizobium* species nodulating soybeans, *B. japonicum*, *B. diazoefficiens*, and *B. elkanii* were widely distributed around the world, whereas *B. liaoningense*, *B. daqingense*, and *B. huanghuaihaiense* were so far mainly reported in Asia (2–6, 8–10, 18, 33, 50). Although *B. yuanmingense* nodulating various legumes has been found in several continents, the biovar nodulating soybeans has so far been found only in Asia (2, 3, 5, 50). The observed high diversity of soybean-nodulating *Bradyrhizobium* in Asia is consistent with the presence of wild soybeans and the long history of soybean cultivation in this region (51, 52).

Molecular diversity of island and off-island genes. In contrast to some strains such as BTAi1 and ORS278, which use a Nod-



(a) *off_island* genes



(b) *island* genes

FIG 2 ML tree of off-island genes (a) and island genes (b). Off-island genes: *SMc00019*, *truA*, *thrA*, *hsfA*, *exoN*, *phyR*, *glyA*, and *fabB*. Island genes: *nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *rhcC2*, and *trpD*. In panel b, cluster III includes *B. yuanmingense* and *B. daqingense* CCBAU 15772, and cluster IV contains the remaining species except *B. elkanii* and *Bradyrhizobium* sp. II. Scale bars indicate 4% substitutions per site.

TABLE 2 Molecular diversity for genetic markers

Gene (length, bp)	No. of seg. sites[a]	*h*/Hd[b]	π[c]	π_S[d]	π_N[e]
Off-island genes					
SMc00019 (367)	108	25/0.952	0.08583	0.29780	0.03567
truA (512)	185	29/0.962	0.10237	0.33936	0.04514
thrA (479)	147	32/0.966	0.08754	0.35149	0.01980
hsfA (437)	138	28/0.956	0.08583	0.26083	0.04514
exoN (571)	150	34/0.969	0.06925	0.22485	0.03087
phyR (422)	109	29/0.957	0.07090	0.29060	0.01183
glyA (631)	160	32/0.969	0.06666	0.23850	0.02106
fabB (572)	195	31/0.968	0.10664	0.36170	0.04172
Avg	149	30/0.962	0.08437	0.29564	0.03140
Island genes					
rhcC2 (598)	159	15/0.762	0.08314	0.26753	0.0335
trpD (622)	204	19/0.831	0.08242	0.18165	0.05786
fixA (579)	147	11/0.678	0.07184	0.20960	0.03068
nifA (585)	186	14/0.586	0.09329	0.22395	0.06165
nifH (620)	115	13/0.659	0.05006	0.22459	0.00380
nodV (647)	145	10/0.627	0.06514	0.20456	0.02974
nodC (568)	132	9/0.555	0.05746	0.18179	0.02090
Avg	155	13/0.671	0.07190	0.21338	0.03401

[a] seg. sites, segregating sites.
[b] Haplotype number (*h*) and haplotype diversity (Hd).
[c] π, average number of nucleotide differences per site between two sequences.
[d] π_S, nucleotide diversity for synonymous substitutions (d_S).
[e] π_N, nucleotide diversity for nonsynonymous substitutions (d_N).

independent pathway to form a symbiosis with *Aeschynomene* species (53), most *Bradyrhizobium* strains use the Nod-dependent strategy and are characterized by the genomic feature that key symbiotic functions are encoded by genes localized in a symbiosis island region (26, 27). However, our understanding of the diversity of genes in this island is limited to *nifH*, *nifD*, and several *nod/nol/noe* genes, such as *nodA*, *nodC*, *nodY*, *nodK*, *nodZ*, *nolL*, and *noeI* (14, 18–20, 54). In this study, island genes *nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *rhcC2*, and *trpD* were sequenced (25, 28–31, 55). As shown in Table 2, the lowest and highest π values are 0.05006 and 0.09329 for *nifH* and *nifA*, respectively. This could be partially explained by the large difference between their corresponding π_N values (0.0038 for *nifH* and 0.0617 for *nifA*) rather than by π_S (0.22459 for *nifH* and 0.22395 for *nifA*). In line with this observation, regulation mechanisms of nitrogen fixation differ in diverse diazotrophs (56). Moreover, GAF domains of different NifA proteins have a role in regulating NifA activity and seem to have a diverse role in various diazotrophs (56). Therefore, in contrast to the highly conserved nitrogenase component protein NifH, nonsynonymous changes of the regulator gene *nifA* could be under a different level of selection pressure. Values of π_N/π_S (d_N/d_S) for *nifA* (0.275) and *trpD* (0.319) are above 0.25, whereas the values for the other test genes are below this boundary, with the value of *nifH* being the lowest (0.017). This further suggested stronger purifying selection acting on *nifH* than on *nifA*. Since the off-island copy of *trpD* has been shown to be essential for tryptophan biosynthesis (57, 58), the sequenced island copy of *trpD* in this study might have been subject to relaxed negative selection for new functionalization.

Compared to off-island genes *SMc00019*, *truA*, *thrA*, *hsfA*, *exoN*, *phyR*, *glyA*, and *fabB* (17, 22–24), those island genes showed significantly lower average values of *h* (*t* test, $P < 0.0001$), Hd ($P = 0.0002$), and π_S ($P = 0.0027$) but no significant differences in π or π_N (*t* test, $\alpha = 0.05$). To avoid the potential effect of the variations among different species on the estimation of the statistics of molecular diversity, off-island and island genes within each species were also compared. Five species with more than eight strains were analyzed here: *B. japonicum* (22 strains), *B. diazoefficiens* (11 strains), *B. yuanmingense* (10 strains), *Bradyrhizobium* sp. III (8 strains), and *B. elkanii* (10 strains). As shown in Table 3, π of off-island genes ranges from 0.00499 to 0.01993. In line with earlier studies on soybean rhizobia from Myanmar, India, Nepal, Vietnam, and eastern North America (5, 18), *B. diazoefficiens* ($\pi = 0.00669$) was found to be among the species with a relatively low level of diversity. Moreover, island genes showed obviously lower π, π_S, and π_N than off-island genes in all these five species. Intriguingly, the ratio between off-island π and island π varies from 2.46 in *B. yuanmingense*, through 3.46 in *Bradyrhizobium* sp. III, 8.92 in *B. diazoefficiens*, and 24.91 in *B. japonicum*, to 332.6 in *B. elkanii*. Similar off-island/island ratios were found for π_S and π_N. Since off-island nucleotide diversity in *B. elkanii* ($\pi = 0.01663$) is comparable to that of *B. japonicum* ($\pi = 0.01794$) and *B. yuanmingense* ($\pi = 0.01993$), and clearly higher than that of *B. diazoefficiens* ($\pi = 0.00669$) and *Bradyrhizobium* sp. III ($\pi = 0.00499$), the extremely low diversity of island genes in *B. elkanii* is unlikely to be caused by sampling bias. Instead, these observations suggest a distinct evolutionary history of island genes in *B. elkanii*. The overall lower diversity of island genes than of off-island genes for soybean *Bradyrhizobium* might be due to the selection pressure from the legume host. However, different legume genera/species may select island sequence variants, and this would lead to a higher diversity of island genes than of off-island genes, as reported for *Bradyrhizobium* sampled from 14 legume genera (54).

In addition to point mutation, recombination is also a source of genetic diversity (59). Recent evolutionary genetics studies have shown that recombination could make a contribution compara-

TABLE 3 Molecular diversity of *Bradyrhizobium* nodulating soybean

Group (no. of strains)	No. of seg. sites[a]	*h*/Hd[b]	π[c]	π_S[d]	π_N[e]
Off-island genes					
B. japonicum (22)	247	11/0.862	0.01794	0.06197	0.00299
B. diazoefficiens (11)	79	9/0.964	0.00669	0.02231	0.00140
B. yuanmingense (10)	214	8/0.956	0.01993	0.07080	0.00288
Bradyrhizobium sp. III (8)	44	6/0.929	0.00499	0.01530	0.00154
B. elkanii (10)	136	6/0.844	0.01663	0.05631	0.00322
Island genes					
B. japonicum (22)	18	11/0.883	0.00072	0.00182	0.00038
B. diazoefficiens (11)	10	6/0.873	0.00075	0.00176	0.00041
B. yuanmingense (10)	144	8/0.956	0.00810	0.02257	0.00311
Bradyrhizobium sp. III (8)	22	5/0.786	0.00144	0.00180	0.00132
B. elkanii (10)	1	2/0.200	0.00005	0.00018	0

[a] seg. sites, segregating sites calculated with all genes used in this study.
[b] Haplotype number (*h*) and haplotype diversity (Hd).
[c] π, average number of nucleotide differences per site between two sequences.
[d] π_S, nucleotide diversity for synonymous substitutions (d_S).
[e] π_N, nucleotide diversity for nonsynonymous substitutions (d_N).

ble to or greater than that of mutation in creating diversity of rhizobia (7, 18, 49). This phenomenon was further supported, in this study, by the *r/m* values of 1.12 ± 0.03 (average ± standard error of the mean [SEM]) and 1.65 ± 0.13 for off-island and island genes, respectively. However, ρ/θ was 0.096 ± 0.009 (average ± SEM) for island genes, which is less than half of the value for off-island genes (ρ/θ = 0.21 ± 0.005), indicating a lower frequency of recombination in island genes than in off-island genes. This is consistent with the significantly lower average number of recombination events (*Rm*) per island gene (*Rm* = 13.7) than the comparable figure for off-island genes (*Rm* = 30.5; *t* test, *P* = 0.00015; see Table S4 in the supplemental material). When we look at the *Rm* values for *B. japonicum*, *B. diazoefficiens*, *B. yuanmingense*, *Bradyrhizobium* sp. III, or *B. elkanii* (Table 4), *Rm* calculated with island genes is always lower than that obtained with off-island genes for the same species. For the off-island genes, the observed *Rm* values for *B. japonicum*, *B. yuanmingense*, and *B. elkanii* lie outside the 95% interval (upper limit) of values obtained with coalescence simulations with an intermediate level of recombination, which is consistent with high rates of recombination within each species. *B. diazoefficiens* and *Bradyrhizobium* sp. III showed an intermediate level of recombination compared to the simulation data. These observed levels of recombination in off-island genes are comparable to or higher than those reported earlier for *B. japonicum*, *B. diazoefficiens*, *B. yuanmingense*, and *B. elkanii* nodulating soybeans (5). The discrepancy could be due to either the higher diversity of test strains in this study or different sets of off-island genes used in the two studies. The *Rm* values calculated from the concatenated sequences include those recombination events between loci. Island loci are much closer together than are off-island loci in this study (Fig. 1), which might lead to biased estimation of recombination events between loci. To exclude this potential bias on *Rm* estimation, the sum of intragenic *Rm* values for off-island or island genes was calculated for each species. The resulting values of five species range from 1 to 17 and 0 to 1 for off-island and island genes, respectively. For each species, off-island genes always showed a higher level of recombination than did island genes.

TABLE 4 Recombination within species

Group (no. of strains)	*Rm*	Coalescence simulation[a]		
		Rm avg	95% confidence interval	$P \leq$ observed *Rm*
Off-island genes				
B. japonicum (22)	20	3.44	1, 7	1.00
B. diazoefficiens (11)	6	4.35	1, 8	0.86
B. yuanmingense (10)	22	9.42	3, 16	1.00
Bradyrhizobium sp. III (8)	5	3.27	0, 7	0.89
B. elkanii (10)	5	0.85	0, 3	1.00
Island genes				
B. japonicum (22)	0	1.07	0, 3	0.29
B. diazoefficiens (11)	0	0.058	0, 2	0.58
B. yuanmingense (10)	4	0.001	0, 0	1.0
Bradyrhizobium sp. III (8)	1	0.0	0, 0	1.0
B. elkanii (10)	0	0.008	0, 0	0.992

[a] Neutral coalescence simulations given the number of segregating sites with an intermediate level of recombination.

Phylogenetic analysis of off-island and island genes. Molecular diversity analyses imply a different evolutionary history for island genes than for the off-island genes. This view was further proved by the results of tree topology comparisons in the maximum likelihood framework (Table 5). All the gene trees of island genes (*nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *rhcC2*, and *trpD*) were significantly different ($P < 0.001$) from the species tree based on the *SMc00019-truA-thrA* concatenate, whereas no significantly incongruent signals could be detected for the gene tree of each off-island gene compared to the species tree ($P > 0.05$). This result is similar to earlier findings on *nifH*, *nodA*, *nodC*, *nodY*, *nodK*, *nodZ*, *nolL*, and *noeI* (14, 18–20), where *Bradyrhizobium* formed a monophyletic clade in the phylogeny of these symbiosis genes but showed a topology that was incongruent with the reference species tree. In the well-resolved ML tree of the concatenated off-island genes (Fig. 2a), 11 genospecies were clearly identified. In contrast, the tested strains formed only four clusters in the ML tree of island genes (Fig. 2b), i.e., cluster I (*B. elkanii*), cluster II (*Bradyrhizobium* sp. II), cluster III (including *B. yuanmingense* and *B. daqingense* CCBAU 15772), and cluster IV, containing the remaining

TABLE 5 SH test of gene phylogeny with the reference phylogeny based on *SMc00019*, *truA*, and *thrA*

Gene	−ln L^a	Difference in −ln L^b	P^c
Off-island genes			
SMc00019	7,374.87	331.96	0.281
truA	7,333.84	290.93	0.314
thrA	7,266.93	224.02	0.373
hsfA	7,600.97	558.07	0.11
exoN	7,477.30	434.40	0.198
phyR	7,699.41	656.50	0.071
glyA	7,459.06	416.15	0.213
fabB	7,658.13	615.22	0.087
All off-island genes	7,111.34	68.48	0.404
Island genes			
rhcC2	15,655.20	8,612.30	0.000*
trpD	13,532.42	6,489.51	0.000*
fixA	14,849.94	7,807.03	0.000*
nifA	15,863.37	8,820.46	0.000*
nifH	14,704.16	7,661.25	0.000*
nodV	14,933.56	7,890.65	0.000*
nodC	15,866.32	8,823.41	0.000*
All island genes	12,205.51	5,162.61	0.000*

[a] −ln *L*, negative log-likelihood values correspond to those for the constrained topology.
[b] Score differences between unconstrained and constrained trees.
[c] Significance of difference in −ln *L* scores achieved by constrained and unconstrained trees, as assessed by SH test. *, $P < 0.05$.

eight genospecies. CCBAU 15772, 15766, and 15773 were isolated from the same sampling site (see Table S1 in the supplemental material), but 15766 and 15772 belong to *B. daqingense* whereas strain 15773 belongs to *B. yuanmingense*. Strain 15772 might have obtained the typical island genes of *B. yuanmingense* from strain 15773, or vice versa. It was hypothesized that dissemination of nodulation and nitrogen fixation genes within the *Bradyrhizobium* lineage mainly occurred through vertical transmission, with a limited role for lateral gene transfer (19, 20). In this study, the grouping of strains belonging to cluster I, II, or III in the island phylogeny (Fig. 2b) closely followed the off-island phylogeny (Fig. 2a). Therefore, in addition to nodulation and nitrogen fixation genes, other genes (such as *fixA*, *rhcC2*, and *trpD*) in the symbiosis island may also be mainly disseminated through vertical transmission in *B. elkanii*, *B. yuanmingense*, and *Bradyrhizobium* sp. II. However, the epidemic cluster IV of island genes in eight genospecies might be viewed as a bona fide example of lateral gene transfer. Moreover, *nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *rhcC2*, and *trpD* could be disseminated together, as supported by the similar phylogeny among these island genes (data not shown). Congruent phylogeny among *nifH* and nodulation genes was also reported in earlier studies of *Bradyrhizobium* (19, 20). The transfer of island genes into certain chromosomal backgrounds has also been observed in *Bradyrhizobium* strains isolated from other legume genera (54). A similar situation has been found with symbiosis plasmids in *Rhizobium* and *Sinorhizobium* species (60–62).

Genetic divergence and gene flow. As island genes are considered to be typical accessory genes, the acquisition of island genes does not require homology to integrate into the recipient genome (63, 64). Therefore, the evolutionary fate of such genes may be only loosely coupled with that of species where they are found (64). However, in the phylogenetic analyses of this study, the epidemic cluster IV of island genes was not found in *B. elkanii*, *B. yuanmingense*, and *Bradyrhizobium* sp. II (Fig. 2b). Could the transmission patterns of island genes be related to the genetic

TABLE 6 Genetic divergence and gene flow between populations

Variable and population	Value for population[e]:				
	B. elkanii	*B. yuanmingense*	*B. japonicum*	*B. diazoefficiens*	*Bradyrhizobium* sp. III
Dxy[a]					
B. elkanii		0.16470	0.17608	0.17612	0.17626
B. yuanmingense	0.14851		0.11565	0.11572	0.11548
B. japonicum	0.15427	0.08286		0.00083	0.00123
B. diazoefficiens	0.14773	0.08248	0.07296		0.00121
Bradyrhizobium sp. III	0.14737	0.07691	0.07361	0.07496	
ANI[b]					
B. elkanii	0.9708/0.9997[c]	0.8361	0.8245	0.8242	0.8242
B. yuanmingense	0.8551	0.9678/0.9695	0.8859	0.8854	0.8857
B. japonicum	0.8485	0.9202	0.9653/0.9976	1	1
B. diazoefficiens	0.8546	0.9197	0.9431	0.9866/0.9980	0.9997
Bradyrhizobium sp. III	0.8546	0.9260	0.9280	0.9270	0.9906/0.995
Nm[d]					
B. elkanii		0.01	0.00	0.00	0.00
B. yuanmingense	0.07		0.02	0.02	0.02
B. japonicum	0.06	0.15		4.28	4.99
B. diazoefficiens	0.04	0.10	0.10		6.07
Bradyrhizobium sp. III	0.04	0.10	0.09	0.04	

[a] *Dxy* is the average nucleotide divergence between groups.
[b] The maximum interspecies ANI values are shown in the upper and lower triangles.
[c] Minimum intraspecies ANI values (off-island/island) calculated with off-island and island genes, respectively.
[d] *Nm*, number of migrants.
[e] The underlined values were calculated with island genes, and the other values were calculated with off-island genes.

divergence level of off-island genes? As shown in Table 6 and Table 3, *Dxy* values (the average nucleotide divergence between genospecies) are all higher than the within-genospecies divergence (π) for off-island genes in representative species, including *B. elkanii*, *B. yuanmingense*, *B. japonicum*, *B. diazoefficiens*, and *Bradyrhizobium* sp. III, reflecting genetic differentiation among these genospecies. However, *Dxy* values calculated for island genes among *B. japonicum*, *B. diazoefficiens*, and *Bradyrhizobium* sp. III were similar to or lower than π for each genospecies. Notably, the *Dxy* values of off-island genes between *B. japonicum*, *B. diazoefficiens*, and *Bradyrhizobium* sp. III were among the lowest values in this study. Moreover, those island *Dxy* values higher than corresponding π values showed a positive linear relationship with off-island *Dxy* values (Pearson coefficient = 0.9898, $P < 0.0001$). When the off-island *Dxy* values were lower than 0.075, island *Dxy* values dropped dramatically (Table 6). Similarly, a clear gap between intraspecies and interspecies ANI values could be observed for those calculated with off-island genes, but no intraspecies/interspecies ANI gaps of island genes were found among *B. japonicum*, *B. diazoefficiens*, and *Bradyrhizobium* sp. III (Table 6). The relationships between island and off-island ANI values were similar to those between island and off-island *Dxy*. The high level of *Nm* calculated with island genes among three less divergent species in off-island genes (*B. japonicum*, *B. diazoefficiens*, and *Bradyrhizobium* sp. III) further supported a potential relationship between the gene flow of island genes and the divergence of off-island genes (Table 6). Taken together, the different levels of gene flow in island genes among different genospecies defined by off-island genes might imply that island genes require the correct off-island background to function. This view was also supported by earlier phylogenetic analyses of nodulation and nitrogen fixation genes in *Bradyrhizobium* and the comparative genomics of soybean rhizobia (7, 19, 20). On the other hand, the cooccurrence of these *Bradyrhizobium* species in sympatry (see Table S3 in the supplemental material) precludes the potential influence of geographic isolation on the observed phenomenon.

Conclusions. Based on three useful phylogenetic and taxonomic markers (*SMc00019*, *thrA*, and *truA*), 76 representative *Bradyrhizobium* strains nodulating soybean in China were grouped into 11 genospecies. This was confirmed by analyses of eight off-island genes (*SMc00019*, *thrA*, *truA*, *fabB*, *glyA*, *phyR*, *exoN*, and *hsfA*). However, island genes (*nifA*, *nifH*, *nodC*, *nodV*, *fixA*, *trpD*, and *rhcC2*) showed characteristics contrasting with those of off-island genes in terms of nucleotide diversity and the rate of recombination. Variations in related statistics were also observed between different island genes (such as *nifA* and *nifH*) or between different genospecies. Although phylogenetic analyses suggested a different evolutionary history of island genes in contrast to off-island genes, variations in the gene flow level of island genes among different genospecies might imply that island genes require the correct off-island background to function.

ACKNOWLEDGMENTS

We thank J. Peter W. Young for language revision and comments.

This study was funded by the National Natural Science Foundation of China (31200002).

REFERENCES

1. **Qiu LJ, Chen PY, Liu ZX, Li YH, Guan RX, Wang LH, Chang RZ.** 2011. The worldwide utilization of the Chinese soybean germplasm collection. Plant Genet. Resour. **9:**109–122. http://dx.doi.org/10.1017/S1479262110000493.
2. **Zhang YM, Li Y, Jr, Chen WF, Wang ET, Tian CF, Li QQ, Zhang YZ, Sui XH, Chen WX.** 2011. Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. Appl. Environ. Microbiol. **77:**6331–6342. http://dx.doi.org/10.1128/AEM.00542-11.
3. **Li QQ, Wang ET, Zhang YZ, Zhang YM, Tian CF, Sui XH, Chen WF, Chen WX.** 2011. Diversity and biogeography of rhizobia isolated from root nodules of *Glycine max* grown in Hebei province, China. Microb. Ecol. **61:**917–931. http://dx.doi.org/10.1007/s00248-011-9820-0.
4. **Shiro S, Matsuura S, Saiki R, Sigua GC, Yamamoto A, Umehara Y, Hayashi M, Saeki Y.** 2013. Genetic diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in the United States. Appl. Environ. Microbiol. **79:**3610–3618. http://dx.doi.org/10.1128/AEM.00236-13.
5. **Vinuesa P, Rojas-Jimenez K, Contreras-Moreira B, Mahna SK, Prasad BN, Moe H, Selvaraju SB, Thierfelder H, Werner D.** 2008. Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. Appl. Environ. Microbiol. **74:**6987–6996. http://dx.doi.org/10.1128/AEM.00875-08.
6. **Zhang YM, Li Y, Jr, Chen WF, Wang ET, Sui XH, Li QQ, Zhang YZ, Zhou YG, Chen WX.** 2012. *Bradyrhizobium huanghuaihaiense* sp. nov., an effective symbiotic bacterium isolated from soybean (*Glycine max* L.) nodules. Int. J. Syst. Evol. Microbiol. **62:**1951–1957. http://dx.doi.org/10.1099/ijs.0.034546-0.
7. **Tian CF, Zhou YJ, Zhang YM, Li QQ, Zhang YZ, Li DF, Wang S, Wang J, Gilbert LB, Li YR, Chen WX.** 2012. Comparative genomics of rhizobia nodulating soybean suggests extensive recruitment of lineage-specific genes in adaptations. Proc. Natl. Acad. Sci. U. S. A. **109:**8629–8634. http://dx.doi.org/10.1073/pnas.1120436109.
8. **Wang JY, Wang R, Zhang YM, Liu HC, Chen WF, Wang ET, Sui XH, Chen WX.** 2013. *Bradyrhizobium daqingense* sp. nov., isolated from soybean nodules. Int. J. Syst. Evol. Microbiol. **63:**616–624. http://dx.doi.org/10.1099/ijs.0.034280-0.
9. **Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Melo IS, Martínez-Romero E, Hungria M.** 2013. Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum group* Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. Int. J. Syst. Evol. Microbiol. **63:**3342–3351. http://dx.doi.org/10.1099/ijs.0.049130-0.
10. **Man CX, Wang H, Chen WF, Sui XH, Wang ET, Chen WX.** 2008. Diverse rhizobia associated with soybean grown in the subtropical and tropical regions of China. Plant Soil **310:**77–87. http://dx.doi.org/10.1007/s11104-008-9631-3.
11. **Yang S, Tang F, Gao M, Krishnan HB, Zhu H.** 2010. R gene-controlled host specificity in the legume-rhizobia symbiosis. Proc. Natl. Acad. Sci. U. S. A. **107:**18735–18740. http://dx.doi.org/10.1073/pnas.1011957107.
12. **Willems A, Coopman R, Gillis M.** 2001. Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium*. Int. J. Syst. Evol. Microbiol. **51:**623–632.
13. **Willems A, Coopman R, Gillis M.** 2001. Phylogenetic and DNA-DNA hybridization analyses of *Bradyrhizobium* species. Int. J. Syst. Evol. Microbiol. **51:**111–117.
14. **Vinuesa P, Silva C, Werner D, Martinez-Romero E.** 2005. Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. Mol. Phylogenet. Evol. **34:**29–54. http://dx.doi.org/10.1016/j.ympev.2004.08.020.
15. **Menna P, Barcellos FG, Hungria M.** 2009. Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. Int. J. Syst. Evol. Microbiol. **59:**2934–2950. http://dx.doi.org/10.1099/ijs.0.009779-0.
16. **Rivas R, Martens M, de Lajudie P, Willems A.** 2009. Multilocus sequence analysis of the genus *Bradyrhizobium*. Syst. Appl. Microbiol. **32:**101–110. http://dx.doi.org/10.1016/j.syapm.2008.12.005.
17. **Zhang YM, Tian CF, Sui XH, Chen WF, Chen WX.** 2012. Robust markers reflecting phylogeny and taxonomy of rhizobia. PLoS One **7:**e44936. http://dx.doi.org/10.1371/journal.pone.0044936.
18. **Tang J, Bromfield ESP, Rodrigue N, Cloutier S, Tambong JT.** 2012. Microevolution of symbiotic *Bradyrhizobium* populations associated with soybeans in east North America. Ecol. Evol. **2:**2943–2961. http://dx.doi.org/10.1002/ece3.404.
19. **Moulin L, Béna G, Boivin-Masson C, Stepkowski T.** 2004. Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene

co-transfer within the *Bradyrhizobium* genus. Mol. Phylogenet. Evol. **30:** 720–732. http://dx.doi.org/10.1016/S1055-7903(03)00255-0.
20. **Menna P, Hungria M.** 2011. Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidences for the theory of monophyletic origin and spread and maintenance by both horizontal and vertical transfer. Int. J. Syst. Evol. Microbiol. **61:**3052–3067. http://dx.doi.org/10.1099/ijs.0.028803-0.
21. **Aserse AA, Räsänen LA, Aseffa F, Hailemariam A, Lindström K.** 2012. Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of *Crotalaria spp.*, *Indigofera* spp., *Erythrina brucei* and *Glycine max* growing in Ethiopia. Mol. Phylogenet. Evol. **65:**595–609. http://dx.doi.org/10.1016/j.ympev.2012.07.008.
22. **Oh HS, Son O, Chun JY, Stacey G, Lee MS, Min KH, Song ES, Cheon CI.** 2001. The *Bradyrhizobium japonicum hsfA* gene exhibits a unique developmental expression pattern in cowpea nodules. Mol. Plant Microbe Interact. **14:**1286–1292. http://dx.doi.org/10.1094/MPMI.2001.14.11.1286.
23. **Gourion B, Sulser S, Frunzke J, Francez-Charlot A, Stiefel P, Pessi G, Vorholt JA, Fischer HM.** 2009. The PhyR-sigma(EcfG) signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. Mol. Microbiol. **73:**291–305. http://dx.doi.org/10.1111/j.1365-2958.2009.06769.x.
24. **Rossbach S, Hennecke H.** 1991. Identification of *glyA* as a symbiotically essential gene in *Bradyrhizobium japonicum*. Mol. Microbiol. **5:**39–47. http://dx.doi.org/10.1111/j.1365-2958.1991.tb01824.x.
25. **Mao C, Qiu J, Wang C, Charles TC, Sobral BW.** 2005. NodMutDB: a database for genes and mutants involved in symbiosis. Bioinformatics **21:**2927–2929. http://dx.doi.org/10.1093/bioinformatics/bti427.
26. **Kaneko T, Maita H, Hirakawa H, Uchiike N, Minamisawa K, Watanabe A, Sato S.** 2011. Complete genome sequence of the soybean symbiont *Bradyrhizobium japonicum* strain USDA6T. Genes **2:**763–787. http://dx.doi.org/10.3390/genes2040763.
27. **Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, Tabata S.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**225–256. http://dx.doi.org/10.1093/dnares/9.6.225.
28. **Fischer HM, Alvarez-Morales A, Hennecke H.** 1986. The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in control of *nif* gene expression and formation of determinate symbiosis. EMBO J. **5:**1165–1173.
29. **Göttfert M, Lamb JW, Gasser R, Semenza J, Hennecke H.** 1989. Mutational analysis of the *Bradyrhizobium japonicum* common *nod* genes and further *nod* box-linked genomic DNA regions. Mol. Gen. Genet. **215:**407–415. http://dx.doi.org/10.1007/BF00427037.
30. **Göttfert M, Grob P, Hennecke H.** 1990. Proposed regulatory pathway encoded by the *nodV* and *nodW* genes, determinants of host specificity in *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. U. S. A. **87:**2680–2684. http://dx.doi.org/10.1073/pnas.87.7.2680.
31. **Krause A, Doerfel A, Göttfert M.** 2002. Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. Mol. Plant Microbe Interact. **15:**1228–1235. http://dx.doi.org/10.1094/MPMI.2002.15.12.1228.
32. **Okazaki S, Zehner S, Hempel J, Lang K, Gottfert M.** 2009. Genetic organization and functional analysis of the type III secretion system of *Bradyrhizobium elkanii*. FEMS Microbiol. Lett. **295:**88–95. http://dx.doi.org/10.1111/j.1574-6968.2009.01593.x.
33. **Han LL, Wang ET, Han TX, Liu J, Sui XH, Chen WF, Chen WX.** 2009. Unique community structure and biogeography of soybean rhizobia in the saline-alkaline soils of Xinjiang, China. Plant Soil **324:**291–305. http://dx.doi.org/10.1007/s11104-009-9956-6.
34. **Terefework Z, Kaijalainen S, Lindstrom K.** 2001. AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. J. Biotechnol. **91:**169–180. http://dx.doi.org/10.1016/S0168-1656(01)00338-8.
35. **Preisig O, Anthamatten D, Hennecke H.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. U. S. A. **90:**3309–3313. http://dx.doi.org/10.1073/pnas.90.8.3309.
36. **Quelas JI, López-García SL, Casabuono A, Althabegoiti MJ, Mongiardini EJ, Pérez-Giménez J, Couto A, Lodeiro AR.** 2006. Effects of N-starvation and C-source on *Bradyrhizobium japonicum* exopolysaccharide production and composition, and bacterial infectivity to soybean roots. Arch. Microbiol. **186:**119–128. http://dx.doi.org/10.1007/s00203-006-0127-3.
37. **Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer HM, Hennecke H.** 2007. Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. Mol. Plant Microbe Interact. **20:**1353–1363. http://dx.doi.org/10.1094/MPMI-20-11-1353.
38. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
39. **Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O.** 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. **59:**307–321. http://dx.doi.org/10.1093/sysbio/syq010.
40. **Posada D, Crandall KA.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818. http://dx.doi.org/10.1093/bioinformatics/14.9.817.
41. **Shimodaira H, Hasegawa M.** 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. **16:**1114–1116. http://dx.doi.org/10.1093/oxfordjournals.molbev.a026201.
42. **Swofford DL.** 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA.
43. **Librado P, Rozas J.** 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25:**1451–1452. http://dx.doi.org/10.1093/bioinformatics/btp187.
44. **Nei M.** 1987. Molecular evolutionary genetics. Columbia University Press, New York, NY.
45. **Nei M, Gojobori T.** 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418–426.
46. **Hudson RR, Slatkin M, Maddison WP.** 1992. Estimation of levels of gene flow from DNA sequence data. Genetics **132:**583–589.
47. **Hudson RR, Kaplan NL.** 1985. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics **111:**147–164.
48. **Didelot X, Falush D.** 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics **175:**1251–1266. http://dx.doi.org/10.1534/genetics.106.063305.
49. **Tian CF, Young JPW, Wang ET, Tamimi SM, Chen WX.** 2010. Population mixing of *Rhizobium leguminosarum* bv. viciae nodulating *Vicia faba*: the role of recombination and lateral gene transfer. FEMS Microbiol. Ecol. **73:**563–576. http://dx.doi.org/10.1111/j.1574-6941.2010.00909.x.
50. **Appunu C, Sasirekha N, Prabavathy VR, Nair S.** 2009. A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera. Biol. Fertil. Soils **46:**57–63. http://dx.doi.org/10.1007/s00374-009-0405-8.
51. **Li Y, Guan R, Liu Z, Ma Y, Wang L, Li L, Lin F, Luan W, Chen P, Yan Z, Guan Y, Zhu L, Ning X, Smulders MJ, Li W, Piao R, Cui Y, Yu Z, Guan M, Chang R, Hou A, Shi A, Zhang B, Zhu S, Qiu L.** 2008. Genetic structure and diversity of cultivated soybean (*Glycine max* (L.) Merr.) landraces in China. Theor. Appl. Genet. **117:**857–871. http://dx.doi.org/10.1007/s00122-008-0825-0.
52. **Wen Z, Ding Y, Zhao T, Gai J.** 2009. Genetic diversity and peculiarity of annual wild soybean (*G. soja* Sieb. et Zucc.) from various eco-regions in China. Theor. Appl. Genet. **119:**371–381. http://dx.doi.org/10.1007/s00122-009-1045-y.
53. **Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Vermeglio A, Medigue C, Sadowsky M.** 2007. Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. Science **316:**1307–1312. http://dx.doi.org/10.1126/science.1139548.
54. **Parker MA.** 2012. Legumes select symbiosis island sequence variants in *Bradyrhizobium*. Mol. Ecol. **21:**1769–1778. http://dx.doi.org/10.1111/j.1365-294X.2012.05497.x.
55. **Göttfert M, Rothlisberger S, Kundig C, Beck C, Marty R, Hennecke H.** 2001. Potential symbiosis-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. J. Bacteriol. **183:**1405–1412. http://dx.doi.org/10.1128/JB.183.4.1405-1412.2001.

56. **Dixon R, Kahn D.** 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2:**621–631. http://dx.doi.org/10.1038/nrmicro954.
57. **Kuykendall LD, Hunter WJ.** 1995. Symbiotic ineffectiveness of *trpCD* deletion mutants of *Bradyrhizobium japonicum*. Soil Biol. Biochem. **27:**1035–1039. http://dx.doi.org/10.1016/0038-0717(95)00013-5.
58. **Kuykendall LD, Hunter WJ.** 1997. The sequence of a symbiotically essential *Bradyrhizobium japonicum* operon consisting of *trpD*, *trpC* and a *moaC*-like gene. Biochim. Biophys. Acta Gene Struct. Expr. **1350:**277–281. http://dx.doi.org/10.1016/S0167-4781(96)00237-0.
59. **Guttman DS, Dykhuizen DE.** 1994. Clonal divergence in *Escherichia coli* as a result of recombination, not mutation. Science **266:**1380–1383. http://dx.doi.org/10.1126/science.7973728.
60. **Guo HJ, Wang ET, Zhang XX, Li QQ, Zhang YM, Tian CF, Chen WX.** 2014. Replicon-dependent differentiation of symbiosis-related genes in *Sinorhizobium* nodulating *Glycine max*. Appl. Environ. Microbiol. **80:**1245–1255. http://dx.doi.org/10.1128/AEM.03037-13.
61. **Ormeño-Orrillo E, Menna P, Almeida LG, Ollero FJ, Nicolás MF, Pains Rodrigues E, Shigueyoshi Nakatani A, Silva Batista JS, Oliveira Chueire LM, Souza RC, Ribeiro Vasconcelos AT, Megías M, Hungria M, Martínez-Romero E.** 2012. Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). BMC Genomics **13:**735. http://dx.doi.org/10.1186/1471-2164-13-735.
62. **González V, Acosta JL, Santamaría RI, Bustos P, Fernández JL, Hernández González IL, Díaz R, Flores M, Palacios R, Mora J, Dávila G.** 2010. Conserved symbiotic plasmid DNA sequences in the multireplicon pangenomic structure of *Rhizobium etli*. Appl. Environ. Microbiol. **76:**1604–1614. http://dx.doi.org/10.1128/AEM.02039-09.
63. **Wiedenbeck J, Cohan FM.** 2011. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiol. Rev. **35:**957–976. http://dx.doi.org/10.1111/j.1574-6976.2011.00292.x.
64. **Fraser C, Alm EJ, Polz MF, Spratt BG, Hanage WP.** 2009. The bacterial species challenge: making sense of genetic and ecological diversity. Science **323:**741–746. http://dx.doi.org/10.1126/science.1159388.
65. **Becker BU, Kosch K, Parniske M, Müller P.** 1998. Exopolysaccharide (EPS) synthesis in *Bradyrhizobium japonicum*: sequence, operon structure and mutational analysis of an *exo* gene cluster. Mol. Gen. Genet. **259:**161–171. http://dx.doi.org/10.1007/s004380050801.