Exploring Genetic Diversity and Signatures of Horizontal Gene Transfer in Nodule Bacteria Associated with *Lotus japonicus* in Natural Environments

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To investigate the genetic diversity and understand the process of horizontal gene transfer (HGT) in nodule bacteria associated with *Lotus japonicus*, we analyzed sequences of three housekeeping and five symbiotic genes using samples from a geographically wide range in Japan. A phylogenetic analysis of the housekeeping genes indicated that *L. japonicus* in natural environments was associated with diverse lineages of *Mesorhizobium* spp., whereas the sequences of symbiotic genes were highly similar between strains, resulting in remarkably low nucleotide diversity at both synonymous and nonsynonymous sites. Guanine-cytosine content values were lower in symbiotic genes, and relative frequencies of recombination between symbiotic genes were also lower than those between housekeeping genes. An analysis of molecular variance showed significant genetic differentiation among populations in both symbiotic and housekeeping genes. These results confirm that the *Mesorhizobium* genes required for symbiosis with *L. japonicus* behave as a genomic island (i.e., a symbiosis island) and suggest that this island has spread into diverse genomic backgrounds of *Mesorhizobium* via HGT events in natural environments.

Furthermore, our data compilation revealed that the genetic diversity of symbiotic genes in *L. japonicus*-associated symbionts was among the lowest compared with reports of other species, which may be related to the recent population expansion proposed in Japanese populations of *L. japonicus*.

Keywords: horizontal gene transfer, rhizobium-legume symbiosis

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Horizontal gene transfer (HGT; also known as lateral gene transfer) is the movement of genetic information between genomes (Gogarten and Townsend 2005; Keeling and Palmer 2008; Ochman et al. 2000; Polz et al. 2013). Recent comparative genomic studies have revealed that HGT has frequently occurred in the prokaryotes (Popa et al. 2011), and that HGT has played important roles in microbial evolution by providing opportunities to improve their adaptability, fitness, and competitiveness (Pál et al. 2005; Polz et al. 2013). Notably, HGT has contributed to the evolution of prokaryote–eukaryote interactions, including virulence and symbiosis: many taxa of pathogens and symbionts have been shown to acquire infection and mutualistic abilities via HGT of mobile genetic elements (MGEs) such as plasmids and genomic islands containing multiple genes involved in their interactions with eukaryotes (Ochman and Moran 2001).

Rhizobia are facultative endosymbionts that form nitrogen-fixing nodules on the root of leguminous hosts (Masson-Boivin et al. 2009), and are known as a remarkable example of bacteria having evolved mutualism with plants via HGT of symbiotic genes (Remigi et al. 2016). In rhizobial genomes, genes essential for nodulation, host recognition, infection, and nitrogen fixation are clustered into genomic islands (i.e., symbiosis islands) (Amadou et al. 2008), which are often located in MGEs, either on large plasmids (Galibert et al. 2001), chromosomal islands (Sullivan et al. 2002), or both (Wang et al. 2014). Such symbiosis islands have spread into many taxa via HGT; consequently, rhizobia are distributed across over 100 species in 14 genera of two classes (α - and β -proteobacteria) (Masson-Boivin et al. 2009; Peix et al. 2015).

The legume-rhizobia mutualism is often highly specific, such that each rhizobial species or strain interacts with specific groups of legumes (Parker 2012; Wang et al. 2012), although the extent of specificity varies by species (Andrews and Andrews 2017). HGT of symbiosis islands has been suggested to play a key role in alterations in these specific interactions, because bacterial lineages sharing the highly similar symbiosis islands often confer nodulation ability with the same legume species (Remigi et al. 2016). To understand how species-specific symbiotic interactions evolve, it is essential to reveal how HGT of symbiosis islands occurred within bacterial strains specific to certain legume species. Although there are a number of studies arguing the signatures of HGT of symbiosis islands, many were based on phylogenetic incongruence between bacterial core genes and symbiotic genes; these studies were often based on a limited number of genes and samples isolated from a geographically restricted area (Bamba et al. 2016; Barcellos et al. 2007; Koppell and Parker 2012; Lemaire et al. 2015; Menna and Hungria 2011; Parker and Rousteau 2014; Steenkamp et al. 2008). However, because HGT often occurs among sympatrically distributed organisms, limited sampling may lead to the underestimation of diversity (Didelot and Maiden 2010; Polz et al. 2013). In addition, phylogenetic incongruence can be caused not only by HGT but also by heterogeneous selective pressures between genes or species (Li et al. 2008); thus, it is insufficient evidence of HGT (Baillly et al. 2007). To detect the signatures of HGT and understand how HGT of specific symbiosis islands can occur across diverse bacterial lineages, not only showing the phylogenetic incongruence, it is also necessary to (i) investigate the large number of symbionts associated with single host species collected from a geographically wide range; (ii) show the strikingly low diversity in symbiotic genes compared with multiple core genes at both synonymous and nonsynonymous sites, indicative of recent HGT rather than purifying selection; and (iii) infer the frequency and impact of recombination in symbiosis islands. Although there are a couple of population genetic studies addressing these issues in symbionts associated with *Medicago* spp. and *Glycine max* (Baillly et al. 2007; Guo et al. 2014), these attempts are still limited, particularly in natural environments.

Here, we focused on the rhizobial symbionts associated with *Lotus japonicus* (Regel) K. Larsen. *L. japonicus* is distributed in the temperate regions of Asia from Pakistan to Japan; the Japanese populations spread from the northernmost (N. 44°) to the southernmost (N. 24°) parts of Japan. In *L. japonicus*, there have been extensive studies on the molecular, physiological, and genomic bases of plant-rhizobia symbiosis (Handberg and Stougaard 1992; Kouchi et al. 2004; Madsen et al. 2010; Maekawa et al. 2009; Nishida et al. 2016, 2018; Soyano et al. 2013; Suzuki et al. 2011; Szczygłowski et al. 1998), and it is regarded as a model species of the family Fabaceae, toward understanding plant-microbe interactions (Bamba et al. 2019; Gentzmittel et al. 2015). Although two rhizobial symbionts, *Mesorhizobium japonicum* MAFF303099 and *M. loti* TONO, have been isolated from *L. japonicus* and their full-genome sequences made available (Kaneko et al. 2000; Shimoda et al. 2016), the level of diversity of associated rhizobial symbionts and the signatures of HGT of symbiosis islands remain unclear. To investigate the genetic diversity of rhizobial symbionts collected from multiple wild *L. japonicus* populations, we used three housekeeping (*recA*, *atpD*, and *dnaK*) and five symbiotic (*nodA*, *nodB*, *nodC*, *noeJ*, and *nifH*) genes. The three housekeeping genes represent bacterial core genomes; *nodA-C* genes are common *nod* genes essential for nodulation in almost all rhizobial species, the *noeJ* gene is involved in the nodulation factor decorations, and the *nifH* gene encodes nitrogenase, which is involved in nitrogen fixation (Mao et al. 2005; Saeki

and Ronson 2014). On the genome of *M. japonicum* MAFF303099, which is one of the reference strains of nodule bacteria isolated from *L. japonicus*, the three housekeeping genes are not close to each other; *nodA-C* genes are within the 10-kb region, the *nifH* gene is 215 kb away from *nodA-C*, and the *noeJ* gene is 92 kb away from *nifH*. *nodA* and *nodC* genes are adjacent and belong to the same operon (Scott et al. 1996) (Supplementary Fig. S1). To gain insight into the evolutionary processes of HGT in the *L. japonicus*-associated symbionts, we specifically addressed the following questions:

- 1) How diverse are the *L. japonicus*-associated symbionts in their core genes and symbiotic genes?
- 2) What are the levels of within- and among-population variation, and is there a geographic trend in diversity?
- 3) Are there signatures of recent HGT of symbiosis islands?
- 4) Within the symbiosis islands, are there any differences in diversity, tree topology, or recombination between common nodulation (*nod*), host recognition-related (*noeJ*), and nitrogen fixation (*nif*) genes?

RESULTS

Nucleotide diversity of nodule bacteria associated with *L. japonicus* in Japan.

We obtained partial sequences of eight genes (three housekeeping genes and five symbiotic genes) from 104 strains associated with *L. japonicus* growing in Japan (Fig. 1; Supplementary Table S1). We then calculated guanine-cytosine (GC) content value and genetic diversity of each gene, which are summarized in Table 1. Three housekeeping genes showed higher GC content (mean 0.657) than those of symbiotic genes (mean GC: 0.600), and these values were comparable with those of *M. japonicum* MAFF303099 (symbiosis island: 0.598, core chromosome: 0.630) (Kaneko et al. 2000). Housekeeping genes showed higher nucleotide diversity π (mean 0.0478) than those of symbiotic genes (mean π : 0.0054). The levels of diversity at both synonymous sites $\pi(s)$ and nonsynonymous sites $\pi(a)$ were also higher in housekeeping genes (mean $\pi(s)$: 0.1697; mean $\pi(a)$: 0.0056) than in symbiotic genes (mean $\pi(s)$: 0.0180, $\pi(a)$: 0.0012). On the other hand, the mean ratio of $\pi(s)$ to $\pi(a)$ [$\pi(a)/\pi(s)$] of symbiotic genes (mean $\pi(a)/\pi(s)$: 0.1123)

Fig. 1. Sampling locations. Nodule bacteria were collected from 14 locations. Location ID, number of collected strains in the parentheses, and location name are indicated for each location. In location 128, indicated by an asterisk (*), two strains obtained from *Lotus corniculatus* were included.

was higher than that of housekeeping genes (mean $\pi(a)/\pi(s)$: 0.0337), although those of *nodA* and *nifH* were smaller than the mean $\pi(a)/\pi(s)$ of housekeeping genes.

An analysis of molecular variance (AMOVA) showed the significant genetic differentiation among populations for all seven genes ($P < 0.001$) (Table 2), with the percentage of variation Φ_{PT} ranging from 32 to 45% (Table 2). There was no obvious difference in Φ_{PT} between housekeeping and symbiotic genes, although both within- and among-population variances of symbiotic genes were smaller owing to lower nucleotide diversity.

Maximum-likelihood tree.

Phylogenetic analyses were performed for each of eight genes using sequences obtained from 104 and two strains isolated from *L. japonicus* and *L. corniculatus*, respectively, in addition to 37 reference strains, including 7 type materials of *Mesorhizobium* spp. Maximum-likelihood (ML) phylogenetic analysis based on three housekeeping genes revealed diverse *Mesorhizobium* strains associated with *L. japonicus* (Supplementary Figs. S2, S3, and S4). The phylogenetic trees reconstructed from all housekeeping genes showed a clear clade of *Mesorhizobium*, while few highly supported clades were observed within the genus. We also found that the housekeeping genes were not clustered in accordance with their host species. Although several haplotypes were closely related to the model strain *M. japonicum* MAFF303099, which was originally isolated from *L. japonicus* collected from Tochigi, Japan (Kaneko et al. 2000), most haplotypes spread in the highly diverged lineages of *Mesorhizobium*.

ML trees of five symbiotic genes showed generally similar topologies with some gene-specific patterns. Most importantly, the strains associated with *L. japonicus* were closely related to

each other (Supplementary Figs. S5, S6, S7, S8, and S9). Three *nod* gene trees showed a single clade containing only *L. japonicus*-associated strains, although the clades of *nodB* and *nodC* trees were not highly supported (bootstrap values: 100 [*nodA*], 58 [*nodB*], and 60 [*nodC*]). In these *nod* gene trees, *L. japonicus*-, all *L. corniculatus*-, and a part of *Biserrula pelecinus*-associated strains formed a highly supported clade (except for the *nodA* gene of *M. loti* NZP 2014) and, in that clade, *L. corniculatus*-associated strains were paraphyletic to the clade of *L. japonicus*-associated strains. In the *noeJ* tree, *L. japonicus*-associated strains were clustered into a single clade that also contains two *L. corniculatus*-associated strains, and *L. corniculatus*-associated strains showing polyphyly. The ML tree of the *nifH* gene showed a very different clustering pattern from *noeJ* and the three *nod* genes: the strains isolated from *L. japonicus* did not form a single clade but, rather, clustered with *L. corniculatus*-associated strains in a clade with relatively weak support (bootstrap value: 59%). The clade of *L. japonicus*- and *L. corniculatus*-associated strains were located as a sister group of two *B. pelecinus* strains. An approximately unbiased (AU) test statistically supported the phylogenetic incongruence between *nod* and *nifH* genes ($P = 0.02$).

Phylogenetic networks.

To identify the phylogenetic cluster that included an implication of recombination, NeighborNet networks for housekeeping and symbiotic genes were constructed, except for the *noeJ* gene, due to a lack of reference sequences (Figs. 2 and 3). Fifty-two multilocus genotypes (MLGs) were identified in the concatenated sequences of three housekeeping genes (*recA*, *atpD*, and *dnaK*). The network analysis of the housekeeping genes showed reticulated structures. The strains isolated in this study spread across the 19 diverse lineages of *Mesorhizobium* that included 10 species based on similarity with the *recA* sequence of type strains (Fig. 2). In all, 3 of the 19 lineages (lineages vi, vii, and ix) were closely related to the strains isolated from *Lotus* spp. (*M. loti* strains), whereas the other 17 consisted only of strains isolated in this study, or of these and isolates from non *Lotus* spp. (lineage xiv).

The NeighborNet analysis of the housekeeping genes did not show clear geographic clusters (Fig. 2). MLGs were not clustered according to their sampling locations. From a single location, we identified one to eight MLGs, which often belonged to different lineages. Only 2 of 52 MLGs (MLG_25 and MLG_5) were found in multiple locations, while the other 50 MLGs were unique to each location, consistent with AMOVA results (Table 2). In all, 11 of the 19 lineages consisted of multiple MLGs obtained from different locations.

The phylogenetic network analysis of symbiotic genes showed a single cluster that consisted of isolates only from *L. japonicus* (Fig. 3A). We identified 57 MLGs (symbiosis gene type [SYM]) from concatenated sequences of four symbiotic genes (*nodA*, *nodB*, *nodC*, and *nifH*). All SYMs isolated from *L. japonicus* were included in a single cluster independent of SYMs isolated from *L. corniculatus* and *B. pelecinus*.

The reticulate structure of symbiotic genes within the *L. japonicus* cluster is shown in Figure 3B. We identified five lineages, each of which consists of 7 to 20 SYMs. The symbiotic genes were similar to the housekeeping genes in that there were no clear geographic structures at both lineage and SYM levels. The geographic origins of each SYM lineage included as many as six to nine locations, which were spatially widespread. We identified one to six SYMs from each single location. Most SYMs were unique to each location, except one genotype (SYM_17), which was found in two locations.

The NeighborNet of three *nod* genes showed less diversity than that of symbiotic gene sets, including *nifH* (Fig. 3C).

Table 1. GC contents and nucleotide diversity for three housekeeping (*recA*, *atpD*, and *dnaK*) and five symbiotic (*nodA*, *nodB*, *nodC*, *nifH*, and *noeJ*) genes of root nodule bacteria associated with *Lotus japonicus*

Gene	Sites	Sites (poly) ^a	GC ^b	π^c	$\pi(s)^d$	$\pi(a)^e$	$\pi(a)/\pi(s)$
<i>recA</i>	424	114	0.681	0.0496	0.1766	0.0023	0.0131
<i>atpD</i>	714	185	0.659	0.0507	0.1761	0.0072	0.0407
<i>dnaK</i>	649	160	0.632	0.0431	0.1565	0.0074	0.0474
<i>nodA</i>	534	12	0.601	0.0032	0.0118	0.0001	0.0085
<i>nodB</i>	598	27	0.593	0.0023	0.0048	0.0015	0.3116
<i>nodC</i>	770	16	0.600	0.0021	0.0067	0.0005	0.0671
<i>nifH</i>	721	34	0.615	0.0138	0.0510	0.0015	0.0286
<i>noeJ</i>	648	17	0.591	0.0054	0.0158	0.0023	0.1456

^a Number of polymorphism sites.

^b GC contents.

^c Nucleotide diversity.

^d Nucleotide diversity of synonymous sites.

^e Nucleotide diversity of nonsynonymous sites.

Table 2. Analysis of molecular variance results for each gene

Gene	SS ^a	MS ^b	Est. Var. ^c	Φ_{PT}^d
<i>recA</i>	804.742	73.158	7.469	0.40321*
<i>atpD</i>	452.061	41.096	4.166	0.39232*
<i>dnaK</i>	604.618	54.965	5.557	0.38831*
<i>nodA</i>	59.789	5.435	0.550	0.38846*
<i>nodB</i>	27.600	2.509	0.240	0.32077*
<i>nodC</i>	36.977	3.362	0.339	0.38451*
<i>nifH</i>	245.836	22.349	2.320	0.43181*
<i>noeJ</i>	70.559	6.414	0.675	0.45861*

^a Sums of squares.

^b Mean squares.

^c Estimate of variance components.

^d An asterisk (*) indicates significance at $P < 0.001$.

Thirty-one *nod* gene types (NODs) were identified using three *nod* genes (*nodA*, *nodB*, and *nodC*) from *L. japonicus* strains isolated in this study. In total, 8 of 31 NOD types were distributed across multiple locations (two to five locations). As with the network analysis of three housekeeping genes and four symbiotic genes, no clear geographic structures were found in the *nod* gene network.

Through the NeighborNet analyses, we found a total of 19 and 5 lineages of housekeeping and symbiotic genes, respectively, although there were no clear genetic associations between them (Table 3). *Mesorhizobium* strains having the SYMs of each symbiotic gene lineage were found to have the MLGs of 6 to 11 lineages of housekeeping genes, and each housekeeping gene lineage had one to five symbiotic gene lineages. In all, 2 of 19 lineages of housekeeping genes had all five lineages of symbiotic genes.

Recombination analyses.

To infer the genealogy of *L. japonicus*-associated symbionts under the influence of recombination events, we performed ClonalFrame analyses (Didelot and Falush 2007) using four gene sets: all genes (designated “ALL”; *recA*, *atpD*, *dnaK*, *noeJ*, *nifH*, *nodA*, *nodB*, and *nodC*); housekeeping genes (designated “HK”; *recA*, *atpD*, and *dnaK*); symbiotic genes (designated “SI”; *noeJ*, *nifH*, *nodA*, *nodB*, and *nodC*); and *nod* genes (designated “NOD”; *nodA*, *nodB*, and *nodC*) of *L. japonicus*-associated symbionts, five reference strains, and *L. corniculatus*-associated symbionts that were obtained in this study.

First, the gene genealogies obtained from ClonalFrame were consistent overall with the results of the ML phylogenetic and NeighborNet analyses (Supplementary Fig. S10). Topologies of the genealogy inferred from ALL, SI, and NOD gene sets were similar with each other; a single clade consisting of *L. japonicus*-associated strains was identified in all genealogies. In those trees, the sister group of the *L. japonicus*-associated clade was always *L. corniculatus*-associated symbionts isolated in this study. Although *L. japonicus*- and *L. corniculatus*-associated strains also constituted a single clade in those trees, *L. corniculatus*-associated strains were paraphyletic. In contrast, the genealogy inferred from HK gene sets showed a highly different topology in terms of polyphyly of *L. japonicus*-associated strains. No clear geographic structures were observed in all of these genealogies.

The times to the most recent common ancestors (TMRCA) of *L. japonicus*-associated symbionts estimated by ClonalFrame showed a greater than three-fold difference between gene sets: TMRCA inferred from HK gene sets was the highest (0.37743), followed by SI (0.15884), ALL (0.14541), and NOD (0.0995).

To infer the number of historical recombination events that have occurred in the *L. japonicus*-associated symbionts, the minimum number of recombination events (Rm) was estimated for each gene, as well as for concatenated sequences of three housekeeping genes, five symbiotic genes, and three *nod* genes using DnaSP 6.10.01 (Rozas et al. 2017). In all, 28 to 50 putative recombination events were detected in each housekeeping gene and 115 events in the concatenated sequences (Table 4). By contrast, only one to six recombination events

Fig. 2. A NeighborNet network of concatenated sequences of housekeeping genes (*recA-atpD-dnaK*). For each multilocus genotype (MLG), the MLG number, [location ID], and number of strains are shown. The operational taxonomic units with asterisks indicate strains obtained from *Lotus corniculatus* in this study.

were detected in each symbiotic gene, 13 events in the concatenated sequence of symbiotic genes, and 6 events in concatenated *nod* genes. The values of three housekeeping genes, two *nod* genes (*nodA* and *nodC*), and all concatenated sequences were

higher than expected based on coalescence simulations with the assumption of an intermediate level of recombination (Table 4).

The ratio of rates at which nucleotides become substituted as a result of recombination and mutation (r/m) and the relative

Fig. 3. NeighborNet networks of symbiotic genes (SYM: *nodA*, *nodB*, *nodC*, and *nifH*) and *nod* gene (NOD: *nodA*, *nodB*, and *nodC*) sequences. The operational taxonomic units (OTUs) with asterisks indicate strains obtained from *Lotus corniculatus* in this study. **A**, A NeighborNet of four symbiotic genes with reference strains. Species names beside OTUs show their host plants. The region with checkered pattern indicates the cluster that consists of *L. japonicus*-associated strains. **B**, A NeighborNet of four symbiotic genes only from *L. japonicus*-associated strains. Each Roman numeral (I to V) indicates the identified lineage. For each SYM, the SYM number, (location ID), and number of strains are shown. **C**, A NeighborNet of three *nod* genes from the *L. japonicus*-associated strains. For each NOD, the NOD number, (location ID), and number of strains are shown.

frequency of recombination to mutation (ρ/θ) in the evolutionary history were estimated using the ClonalFrame program. The mean ρ/θ values of three housekeeping genes, five symbiotic genes, three *nod* genes, and all genes were 2.436077, 0.004582, 0.004492, and 0.067451, respectively (Table 4). Furthermore, the mean values of r/m estimated from housekeeping genes, symbiotic genes, *nod* genes, and all genes were 6.428082, 0.208664, 0.039835, and 0.537097, respectively. Except for the ALL gene set, r/m , ρ/θ , and Rm values showed a similar tendency: housekeeping genes > symbiotic genes > *nod* genes.

We performed the same analyses using several types of subset samples: “Subgroups” (four subgroups inferred from the genealogy of ALL gene sets), “Populations” (eight subsets that consist of strains collected from the same location), and “Gene sets” (five gene sets of SI, excluding one of the five SI genes). In those subsets, r/m and ρ/θ are generally higher in the HK gene set than in the SI gene set (Supplementary Table S2), indicating that recombination events were more frequent and contributed to greater genetic diversification in housekeeping genes compared with symbiotic genes. The MRCA of HK gene sets was generally more ancient compared with the SI gene sets, while the ALL gene set showed slightly higher values of TMRCA compared with the HK gene set in Population 127 and the Subgroup 3.

DISCUSSION

We explored the diversity of nodule bacteria associated with the model plant *L. japonicus* collected from a geographically

wide range of natural populations in Japan. Although *Lotus* spp. have been known to associated with the nodule bacteria belonging to the genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, and *Bradyrhizobium* (Andrews and Andrews 2017), *L. japonicus* has thus far been reported to associate with only two lines of *Mesorhizobium*: *M. japonicum* MAFF303099 (Kaneko et al. 2000) and *M. loti* TONO (Shimoda et al. 2016). Here, we revealed, from the analyses of three housekeeping genes, that *L. japonicus* plants in natural environments are associated with far more diverse lineages of *Mesorhizobium* spp. (Fig. 2).

Despite this high level of diversity in housekeeping genes, sequences of symbiotic genes appeared to be strikingly similar among *L. japonicus*-associated strains; mean synonymous π of housekeeping genes were about five times higher than those of symbiotic genes (Table 1). There would be two scenarios to explain the marked difference between housekeeping and symbiotic genes: recent HGT of symbiotic genes or stronger purifying selection for symbiotic genes. If purifying selection owing to stronger functional constraints for symbiotic genes plays an important role, the ratio of $\pi(a)/\pi(s)$ would be expected to be much lower in symbiotic genes than in housekeeping genes; however, $\pi(a)/\pi(s)$ were found to be comparable (mean $\pi(a)/\pi(s)$ for housekeeping genes: 0.0337 and for symbiotic genes: 0.1123), suggesting that, although $\pi(a)/\pi(s)$ values are <1 in both gene sets, purifying selection is insufficient to explain the low level of diversity in symbiotic genes. We note that our $\pi(a)/\pi(s)$ value of symbiotic genes represented by *nod/noe* and *nifH* genes may be slightly underestimated. This is because

Table 3. Correspondence of housekeeping gene (multilocus genotype [MLG]) and symbiosis gene (SYM) lineages^a

Sym	MLG																	
	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv	xv	xvi	xvii	xviii	xix
I	3	0	16	0	3	0	0	0	0	0	0	0	1	1	1	1	0	0
II	1	0	0	0	3	3	1	0	0	1	0	2	0	0	0	0	1	0
III	3	8	0	1	7	2	1	1	0	1	3	9	0	0	0	1	0	0
IV	3	0	0	2	2	0	0	0	0	0	0	0	1	0	3	0	0	3
V	1	2	1	0	3	1	0	0	2	0	0	0	3	0	0	0	0	2

^a Each row and column shows SYM lineages (I to V) and MLG lineages (ii to xix), respectively. Each cell shows the number of strains with particular SYM and MLG lineages. MLG lineage i was removed because it was obtained from only the *L. corniculatus*-associated strain.

Table 4. Minimum recombination events and impacts of recombination

Gene sets, genes	Rm ^b	Coalescent simulation ^a			r/m^f	ρ/θ^g
		Rm Avg. ^c	95% CI ^d	$P < \text{obs. Rm}^e$		
Sets						
All	128	28.847	19.5–38	1	0.537097	0.067451
Housekeeping	115	19.9090	12.5–29	1	6.428082	2.436077
Symbiosis	13	7.74500	4–12	0.9930	0.208664	0.004582
Nod	6	0.0010	0	1	0.039835	0.004492
Genes						
<i>recA</i>	28	18.6100	12–25	0.997	...	...
<i>atpD</i>	50	12.9360	7–19	1	...	...
<i>dnaK</i>	36	8.4150	4–13	1	...	...
<i>nodA</i>	2	0.0000	0	1	...	...
<i>nodB</i>	1	3.3510	1–7	0.1033	...	...
<i>nodC</i>	1	0.8810	0–3	0.7868	...	...
<i>nifH</i>	6	6.3980	3–11	0.4527	...	...
<i>noeJ</i>	1	3.1100	1–6	0.1320	...	...

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

^b Minimum recombination (Rm) events.

^c Average Rm.

^d 95% Confidence interval (CI) of Rm.

^e P value of estimate Rm < observed Rm.

^f Relative impact of recombination.

^g Relative frequency of recombination. Housekeeping genes: *recA*, *atpD*, and *dnaK*, Symbiosis genes: *nodA*, *nodB*, *nodC*, *nifH*, and *noeJ*. Nod genes: *nodA*, *nodB*, and *nodC*.

Zhang et al. (2014) suggested that the *nifH* gene was relatively conserved among the nitrogen fixation genes (Parker 2012), although the $\pi(a)/\pi(s)$ value of the *nifH* gene would still be comparable with that of housekeeping genes. Rather, it is more likely that HGT occurred recently among diverse lineages of *Mesorhizobium* bacteria, resulting in the striking reduction of the nucleotide variation. The phylogenetic incongruence between housekeeping and symbiotic genes also supports this notion.

In addition, recombination analysis supports the scenario in which the symbiotic genes behave as a genomic island and are transmitted horizontally into diverse genomic backgrounds: the relative frequency (p/θ) and relative impact of recombination (r/m) among the five symbiotic genes were remarkably lower than those of housekeeping genes (Table 4). Our analyses of subsets showed that such patterns would not simply be explained by possible artifacts, including genetic or population structures and uneven representation of genotypes (Smith et al. 2000).

Altogether, these results suggest that symbiosis islands containing genes required for nitrogen-fixing symbiosis with *L. japonicus* spread over diverse lineages of *Mesorhizobium* through HGT. Although the arguments about HGT in most of the previous studies were based on the phylogenetic incongruence between bacterial core genes and symbiotic genes (Barcellos et al. 2007; Koppell and Parker 2012; Lemaire et al. 2015; Menna and Hungria 2011; Parker and Rousteau 2014; Steenkamp et al. 2008), here, we provide multiple lines of evidence consistently supporting the HGT of the symbiosis island: restricted recombination in the symbiotic genes, marked decline in synonymous and nonsynonymous nucleotide diversity compared with genomic backgrounds, and phylogenetic incongruence between housekeeping and symbiotic genes. Several attempts have been made to explore the level of diversity and recombination of housekeeping and symbiotic genes of plant-associated nodule bacteria (Fig. 4; Supplementary Table S3) (Andrus et al. 2012; Cao et al. 2017; Granada et al. 2015; Guo et al. 2014; Harrison et al. 2017; Hollowell et al. 2016; Horn et al. 2014; Ji et al. 2015, 2019; Kumar et al. 2015; Lemaire et al. 2015; Parker 2015; Parker et al. 2015; Pérez-Carrascal et al. 2016; Rashid et al. 2014; Sbabou et al. 2016; Tang et al. 2012; Tian et al. 2010, 2012; Toro et al. 2016; Van Cauwenberghe et al. 2014; Wang et al. 2018; Yao et al. 2015; Zhang et al. 2014). Herein, we

compiled those studies to compare the level of polymorphism with our data from *L. japonicus*-associated symbionts (Fig. 4). Although the nucleotide diversity of housekeeping genes (chromosome π) in *L. japonicus*-associated symbionts was comparable with other studies, π of symbiotic genes (symbiotic π) appeared to be among the lowest in this compiled dataset. Consequently, the relative diversity of symbiotic genes controlled by the genomic background (chromosome π) was lowest among those species. It is worth noting that *L. japonicus*-associated strains, which were obtained from a geographically wide range of natural populations, showed a level of relative diversity in symbiotic genes (symbiotic π /chromosome π) similar to the bacteria associated with *Phaseolus vulgaris* and *G. max* from agricultural fields, which would presumably represent more uniform environments (Guo et al. 2014; Pérez-Carrascal et al. 2016). In summary, compared with other studies, a striking feature of the *L. japonicus*-associated symbionts is their extremely low level of diversity in symbiotic genes, whereas the genomic background represented by housekeeping genes is comparable with other species. We also note that the relative frequency (p/θ) and relative impact of recombination (r/m) were much lower in symbiotic genes compared with housekeeping genes in *L. japonicus*-associated symbionts (Table 4). Although a similar pattern was observed in other systems such as *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium*, the frequency and impact of recombination rates within symbiosis islands relative to those of housekeeping genes were strikingly low compared with previous studies (Guo et al. 2014; Ji et al. 2015, 2019; Wang et al. 2018; Zhang et al. 2014). This result would not be due to simple artifacts by population structure, because the same pattern was consistently observed when analyzing the various subsets.

What makes these unique patterns in symbiotic genes of *L. japonicus*-associated bacteria? We speculate that the demographic history of *L. japonicus* may partly account for this. *L. japonicus* is distributed in the temperate regions of Asia from Pakistan to Japan; the Japanese populations are on the edge of its distribution range and are suggested to derive from the Eurasian continent populations (Gentzbittel et al. 2015). Population genetic studies of Japanese *L. japonicus* suggested that the species has experienced recent population expansion,

References	Host plants	Genus	Chromosome π (mean)	Symbiotic π (mean)	Symbiotic π / Chromosome π
This study	<i>Lotus japonicus</i>	<i>Mesorhizobium</i>	0.0477	0.0053	0.1118
Pérez-Carrascal et al. 2016	<i>Phaseolus vulgaris</i>	<i>Rhizobium</i>	0.0688	0.0093	0.1348
Guo et al. 2014	<i>Glycine max</i>	<i>Sinorhizobium</i>	0.2331	0.0410	0.1759
Andrus et al. 2012	<i>Mimosa</i>	<i>Cupriavidus</i>	0.0230	0.0110	0.4783
Parker 2015	<i>Mimosa</i>	<i>Cupriavidus</i>	0.0280	0.0138	0.4929
Hollowell et al. 2016	<i>Acmispon strigosus</i>	<i>Bradyrhizobium</i>	0.0375	0.0200	0.5333
Tang et al. 2012	<i>Glycine max</i>	<i>Bradyrhizobium</i>	0.0309	0.0188	0.6094
Sbabou et al. 2016	<i>Vicia ervilia</i>	<i>Rhizobium</i>	0.0353	0.0240	0.6792
Ji et al. 2015	<i>Caragana sp.</i>	<i>Mesorhizobium</i>	0.0651	0.0491	0.7545
Zhang et al. 2014	<i>Glycine max</i>	<i>Bradyrhizobium</i>	0.0844	0.0719	0.8522
Cauwenberghe et al. 2014	<i>Vicia cracca</i>	<i>Rhizobium</i>	0.0285	0.0250	0.8772
Toro et al. 2016	<i>Medicago sativa</i>	<i>Ensifer</i>	0.0001	0.0001	1.0296
Tian et al. 2010	<i>Vicia faba</i>	<i>Rhizobium</i>	0.0319	0.0393	1.2296
Parker et al. 2015	<i>Hylodesmum glutinosum</i>	<i>Bradyrhizobium</i>	0.0160	0.0230	1.4375
Horn et al. 2014	<i>Cytisus scoparius</i>	<i>Bradyrhizobium</i>	0.0592	0.0950	1.6047
Parker et al. 2015	<i>Desmodium perplexum</i>	<i>Bradyrhizobium</i>	0.0430	0.0920	2.1395
Granada et al. 2015	<i>Lupinus albescentis</i>	<i>Bradyrhizobium</i>	0.0580	0.1280	2.2078
Parker et al. 2015	<i>Desmodium paniculatum</i>	<i>Bradyrhizobium</i>	0.0570	0.1410	2.4737
Parker et al. 2015	<i>Desmodium canadense</i>	<i>Bradyrhizobium</i>	0.0570	0.1460	2.5614
Parker et al. 2015	<i>Hylodesmum nudiflorum</i>	<i>Bradyrhizobium</i>	0.0190	0.0720	3.7895

Fig. 4. Nucleotide diversity of chromosomal and symbiotic genes compared with previous studies. Reference data were obtained from five genera of nodule bacteria associated with 16 leguminous taxa. Nucleotide diversity π was taken from reference articles. For the definitions of chromosomal and symbiotic genes, we followed each reference article.

colonizing northern Japan after the last ice age (Kai et al. 2010; Mimura 2013; Shah et al. 2018). Therefore, given the low level of polymorphism of symbiotic genes and the contrasting high diversity of the genome background, it is possible that endemic *Mesorhizobium* bacteria in local populations of Japan acquired the ability to associate with *L. japonicus* by recent HGT of symbiotic genes (Nandasena et al. 2006; Sullivan et al. 1995), perhaps in conjunction with the recent spread of *L. japonicus* in the Japanese archipelago. This scenario is also supported by our finding from ClonalFrame analyses that the TMRCAs of symbiotic genes (0.15884) was more recent than that of housekeeping genes (0.37743).

Our analyses of recombination and nucleotide diversity reveal that genes on the symbiosis islands would have different evolutionary histories. Phylogenetic incongruence between *nifH* and other genes ($P = 0.02$; AU test) suggests that recombination occurred within symbiosis islands. Furthermore, higher nucleotide diversity of *nifH* compared with other genes, as well as higher *r/m* values of five symbiotic genes compared with the gene set excluding *nifH*, indicate that recombination of the *nifH* gene occurred between more diverged lineages than that of *nod* and *noeJ* genes. The signature of increased recombination between *nifH* and other genes would not simply be explained by the genomic coordinates of these genes: the location of the *nifH* gene is between *nodA-C* and *noeJ* genes in the symbiosis island of *M. japonicum* MAFF303099. Based on these findings, we propose a scenario in which multiple recombination events between preexisting alleles within symbiosis islands may have contributed to generating adaptive sets of genotypes that spread by recent HGT into *L. japonicus*-associated bacteria. Now that full-genome resequence data have become available for more than 100 accessions of the model plant *L. japonicus* (Shah et al. 2018), the combination of natural accessions of *L. japonicus* and its associated *Mesorhizobium* bacteria discovered in this study would be a valuable model system to address how genetic variations in symbiosis islands may be related to host specificity or host preference.

MATERIALS AND METHODS

Sampling, isolation of nodules, and DNA extraction.

We sampled nodules of *L. japonicus* plants from 14 locations in Japan from 2016 to 2017 (Fig. 1). We sampled 1 to 11 nodules from each individual plant, and two to seven individual plants were surveyed at each site. In total, 104 nodules were obtained from 44 individual plants. All collected nodule samples were used in this study. We also sampled two nodules from one *L. corniculatus* individual that was growing sympatrically with *L. japonicus* at the Aizu population.

Prior to the isolation of rhizobia, nodules were surface sterilized with 70% ethanol for 3 min and 2% sodium hypochlorite for 3 min. The nodules were rinsed three times with sterile distilled water and crushed with a pestle. Crushed nodules were streaked onto a tryptone yeast agar plate and incubated for 4 days at 30°C. After incubation, cloning with the single colonization on agar plate was performed twice. We acknowledge that there may be a sampling bias in this study, because we only used strains that were successfully cultured from nodules. Isolates were stored at -80°C in 30% glycerol for preservation until DNA extraction experiments. From each isolate, DNA was extracted by the cetyltrimethylammonium bromide DNA extraction method (Doyle and Doyle 1987).

Amplification and sequencing.

We amplified partial sequences of three chromosomal housekeeping genes (*recA*, *atpD*, and *dnaK*) and five symbiotic genes (*nodA*, *nodB*, *nodC*, *noeJ*, and *nifH*). *recA*, *atpD*, and

dnaK encode enzymes that are essential for the maintenance of basic cellular functions of bacteria. *nodA*, *nodB*, and *nodC* are common *nod* genes, which are essential for nodulation in almost all rhizobial species and involved in the biosynthesis of Nod factors, determinants of host specificity of nodule bacteria (Perret et al. 2000). *noeJ* gene is involved in the nodulation factor decorations. *nifH* encodes nitrogenase reductase, which is involved in the fixation of atmospheric nitrogen.

PCR was performed using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, U.S.A.). Primers and PCR protocols are described in Supplementary Tables S4 and S5. Amplified DNA fragments were purified using the ExoStar PCR and Sequencing cleanup kit (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), per the manufacturer's instructions. PCR-amplified fragments were sent to Macrogen (Seoul, Korea) for sequencing. Sequences were analyzed and assembled with GeneStudio 2.2.0.0 (GeneStudio Inc., Suwanee, GA, U.S.A.). All sequences were deposited in the GenBank/ENA/DBJ database.

Genetic diversity, genetic variation, and phylogenetic analysis.

Multiple nucleotide sequence alignments were generated with MAFFT 7.245 (Katoh and Standley 2013) with the L-INS-i algorithm. Haplotypes were determined for each gene, based on nucleotide substitutions and indels. Nucleotide diversity of each gene was calculated using DnaSP 6.10.1 (Rozas et al. 2017). Genetic variation of each gene was partitioned among and within different *L. japonicus* populations using a hierarchical AMOVA (Excoffier et al. 1992; Huff et al. 1993), as implemented in Genalex 6.503 (Peakall and Smouse 2012). The significance of genetic differentiation was tested using 9,999 permutations. Populations in which more than three isolates were collected were used for AMOVA.

The phylogenetic tree of each gene was constructed using the ML method with the program GARLI (Zwickl 2006), together with reference sequences obtained from the GenBank database, which are listed in Supplementary Table S6. We used gene sequences isolated from 34 *Mesorhizobium* and 3 *Rhizobium* accessions, in which whole genomic sequences were available, as reference sequences of all following analyses because a BLAST search revealed that all 106 strains isolated in this study belonged to the genus *Mesorhizobium*. Because 32 of those accessions lacked the *noeJ* gene, five reference strains were included in the *noeJ* tree. The nucleotide substitution models were selected by the Akaike Information Criterion, as implemented in jmodeltest 2.1.7 (Darriba et al. 2012; Guindon and Gascuel 2003). The run was terminated after 100,000 generations without an improvement of 0.00001 or more log-likelihood. The single most likely tree out of 10 search replicates was saved for phylogenetic analyses. The robustness of the topologies of the ML tree was inferred by 1,000 bootstrap repetitions. Furthermore, to test phylogenetic congruencies between three *nod* and *nifH* genes, an AU test was performed using CONSEL (Shimodaira and Hasegawa 2001).

To display sequence diversity and visualize recombination events as a NeighborNet network, we first generated concatenated sequence sets for three housekeeping genes (MLG), four symbiotic genes (SYM), and three *nod* genes (NOD). Concatenated sequences were imported into SplitTree 4.12.8 (Huson and Bryant 2006), along with the same sequences used for ML phylogenetic analysis.

We inferred the taxonomic affiliation for each isolate based on the blast hit of the *recA* gene against those of the type materials in the NCBI taxonomic database. We used the *recA* gene because sequence information for most *Mesorhizobium* type strains was available only in this gene, among the three

housekeeping genes that we analyzed. This taxonomic identification should be interpreted with caution because a classification based on a single gene does not provide reliable taxonomic affiliations, given the extensive recombination between genes.

Recombination analysis.

To estimate the R_m , four gamete tests (Hudson and Kaplan 1985) and coalescent simulations were performed with 1,000 replicates using DnaSP 6.10.1 (Rozas et al. 2017). The R_m was estimated for each gene, as well as for three gene sets that were the same as the datasets for NeighborNet analysis (HK: *recA*, *atpD*, and *dnaK*; SI: *nodA*, *nodB*, *nodC*, *noeJ*, and *nifH*; and NOD: *nodA*, *nodB*, and *nodC*).

To assess the effect of recombination, we calculated two parameters: p/θ (the relative frequency of recombination) and r/m (the relative impact of recombination) using ClonalFrame v1.2 (Didelot and Falush 2007). For each dataset, three to five independent runs were performed, each consisting of 2,000,000 iterations. The first 1,000,000 iterations were considered the burn-in period and were discarded. Trees were sampled every 100 iterations, resulting in a sample size of 10,000 from the posterior. Satisfactory Markov Chain Monte Carlo convergence was judged following the criterion of Gelman and Rubin (1992). The analysis was performed using only unique genotypes, because the clonal expansion of specific bacterial genotypes may result in underestimation of recombination events (Smith et al. 2000). In the *L. japonicus*-associated strains used in this study, 58 genotypes were identified in the ALL gene set, 49 genotypes (SI) were identified in the SI gene sets, and unique genotypes of HK and NOD gene sets were the same as those used in the NeighborNet analyses. To deal with the artifacts of genetic and population structure, we also performed the same analyses using several types of subset samples: Subgroups (four subgroups inferred from the genealogy of ALL gene sets), Populations (eight subsets that consist of strains collected from the same location), and Gene sets (five gene sets of SI, excluding one of the five SI genes).

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